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ATTACHMENT B

Declaration under Rule 132 of Dr. David Alexander Clark

DR. DAVID A. CLARK, MD, PhD, FRCP(C), FRCP Edin
Emeritus Professor of Medicine, McMaster University
Consultant in Medical Oncology / Reproductive Medicine
McMaster University Medical Centre
Room HSC-3V39
1200 Main Street West
Hamilton, Ontario
L8N 3Z5

Hamilton Health Sciences

Telephone: (905) 521-2100 Ext. 76376
Fax: (905) 521-4971
Email: clarkd@mcmaster.ca

IN THE MATTER OF
US Patent Application No.
09/380,327 by ROBERTSON et al.

Declaration under U.S.C.§ Rule 132

STATUTORY DECLARATION

I, David Alexander Clark of 444 Smith Ave., Burlington in the Province of Ontario, Canada, do solemnly and sincerely declare as follows:

1. I am the same David Alexander Clark who made statutory declarations dated 16th December 2002 and 9th September 2003 in respect of this matter.

2. I attended an interview at the United States Patent and Trademark Office on 22nd April 2004 with the Examiner, Dr Phuong N. Huynh and the Supervisory Examiner, Ms Christina Chan, which was also attended by Mr. B. Aaron Schulman, the applicant's attorney, and by Mr. Craig Humphris, representing the licensee of the applicant's assignee.

3. At this interview I discussed the prior art documents by Feinberg, Chaouat et al and Clark et al which had been cited by the Examiner. I maintain the opinions about these references which were set out in this discussion, and in my earlier declarations, namely that these references do not disclose or suggest the claims of the above application, and that one of ordinary skill in this art would not have combined these references in order to arrive at the claimed invention on the basis of the teachings in the art at the time of the invention. Moreover, as discussed at the interview, and as explained further below, the cited prior art references actually *teach away* from the present invention, because on the basis of these references and of the teachings at the time of the invention, it would have been thought that administering TGF-β to a prospective mother, either before attempted conception or after attempted conception, would *cause* miscarriage, rather than prevent it.

4. Feinberg showed that TGF-β enhanced production of fibronectin from full-term human placental cytotrophoblast cells, and that the fibronectin could enhance sticking of the cells to a culture dish *in vitro*. Prior to 1997 it was known that activation of latent TGF-β by trophoblasts inhibited trophoblast migration (Graham et al.: Localization of transforming growth factor-β at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. 1992;46:561-572). Indeed, fibrin/fibronectin inhibits trophoblast migration. Prior to 1997 it was also known that reduced trophoblast migration was a characteristic of the histopathology of first trimester miscarriages and of pre-eclampsia (Khong TY et al.: Defective

haemochorial placentation as a cause of miscarriage: a preliminary study. Brit. J. Obstet. Gynaec. 1987;94:649-655). Copies of these publications are now shown to me and annexed hereto as Exhibits DAC-1 and DAC-2.

5. The Feinberg patent discloses a method for achieving improved trophoblast implantation, regardless of whether or not the prospective mother lacks immune tolerance to any paternal antigens. Feinberg is completely silent regarding any suggestion that the presently-claimed method could be used to treat infertility of any kind in a prospective mother who lacked such immune tolerance. There is no reason on the basis of the Feinberg disclosure to suspect any direct beneficial effect of TGF-β on the induction of immune tolerance, and the role of local TGF-β molecules in the genital tract which is described in the present specification would therefore appear to someone skilled in the art to be completely *opposite* to what is suggested in Feinberg. The present method is directed to a set of conditions which is not disclosed or suggested in the Feinberg patent.

6. As discussed at the interview, the paper by Chaouat et al cited by the Examiner described reducing the abortion rate by administering third party BALB/c spleen cells which carried paternal DBA/2 Class I antigens. This protection was ascribed by Chaouat et al to the activation of placental cells to suppress maternal natural killer (NK) cells in the uterus, and to a serum factor reactive with BALB/c and DBA/2 cells, which was found to transfer protection against abortion.

7. Firstly, it would have been readily understood at the priority date of the present application by a person skilled in the art that induction of an immune response such as antibody production is not induction of tolerance; it is induction of an immune response. Secondly, the placental cell-mediated suppression of natural killer cell activity described in this citation was subsequently found to be an artefact caused by increased numbers of foetal erythrocytes in healthier embryos (Clark & Chaouat, Cellular Immunology 1986;102:43-51). A copy of this publication is now shown to me and annexed hereto as Exhibit DAC-3. Thirdly, although Chaouat et all found that CBA/J anti-BALB/c serum could confer protection against abortions in DBA/2-mated CBA/J mice, they admitted that the experiments, in which spleen cells from different strains of mice did or did not absorb out the protective activity, did not prove that the protection was mediated by an anti-H-2d Class I MHC antibody. Antibodies to H-2d induced in CBA/J females by immunizing with DBA/2 cells in this paper did not confer protection. There were several unexplored mechanisms whereby CBA anti-BALB/c serum could have induced protection, and subsequent studies by Chaouat et al attributed the protection primarily to activation of maternal $CD8^+$ T suppressor cells.

8. In fact, attempts to use monoclonal anti-MHC Class I antibodies to protect against abortion in mice were unsuccessful (personal communication to me from Chaouat). This finding teaches away from any suggestion that MHC class I antigens might be useful in eliciting protection against abortion. Moreover, Chaouat did *not* indicate that there was any requirement that the BALB/c splenocytes were male cells; *female* BALB/c cells worked just as well, as was reported in the paper and subsequently confirmed at scientific meetings and in the scientific

literature. In other words Chaouat et al does not suggest in any way that immunization with paternal cells was *essential*.

9. Moreover the results of Chaouat et all suggest that immunization requires an *active* immune response (which was shown to be mediated by $CD8^+$ T cells of the mother) which is completely opposite to the induction of tolerance. Tolerance would mean absence of a response, and anti-MHC antibodies would function in this setting by blocking maternal T cell recognition and reaction to paternal Class I MHC antigens. Thus TGF-β would have been expected to *inhibit* the maternal $CD8^+$ T cell response.

10. There were, therefore, good reasons for a person skilled in the art *not* to use TGF-β together with cells bearing paternal antigens. Since during the period leading up to 1997 there were many people skilled in the art trying to understand how immunotherapy against early abortion might work, I consider that if this combination had been thought likely to be beneficial, it would have been tested.

11. Because of these findings, I consider that a person skilled in the art, prior to the making of the present invention, would have predicted that administration of TGF-β to a prospective mother, either before attempted conception or after attempted conception, *would actually cause miscarriage*. In addition, apart from the possible teratogenic effects of extraneous insults during early embryogenesis, such treatment would be predicted to present a risk of foetal growth restriction, with all of its adverse consequences.

12. Accordingly, one skilled in the art at the time of the present invention would *not* have contemplated combining the disclosures of Feinberg, Chaout and Clark, because it would have been expected that administering TGF-β to a prospective mother, either before attempted conception or after attempted conception, would *cause* miscarriage.

13. To my surprise, as predicted by the inventors in the specification in the present application, I have demonstrated that administration of TGF-β to a prospective mother who lacks immune tolerance to paternal antigen, either before attempted conception or after attempted conception, actually *prevents* miscarriage, and had no detrimental effect on the mother or the fetus during the period of observation. In fact this result was confirmed in the experiment described below.

14. On the basis of these findings I consider that, once in possession of the present specification, I would not expect that there was any limitation on the time at which the TGF-β should be administered. The increased uterine GM-CSF expression stimulated by TGF-β in the experiments reported in the present specification does not imply that the TGF-β should be present in the lumen of the genital tract at the same time as the embryo, nor that it should have to be intraluminal.

15. At the interview I also presented the results of an experimental study which I had performed to evaluate the effect of TGF-β3 on the abortion rate in a mouse model of recurrent

miscarriage. This is described in further detail below. The methods and the mouse model used in this study were widely available at the priority date of the current patent application. When CBA/J mice are mated with DBA/2 mice, the progeny develop in a placental environment which is quantitatively or qualitatively deficient in the production of the anti-inflammatory Th2-type cytokines IL-4 and IL-10, while there are increased levels of local inflammatory cytokines; these factors result in a high incidence of spontaneous foetal resorption (miscarriage). Details concerning the roles of these cytokines in the model were first published in 1990 (Chaouat G, Menu E, Clark DA, Dy M, Minkowski M, Wegmann TG. Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. J Reprod Fertil. 1990; 89:447-58). A copy of this publication is now shown to me and annexed hereto as Exhibit DAC-4.

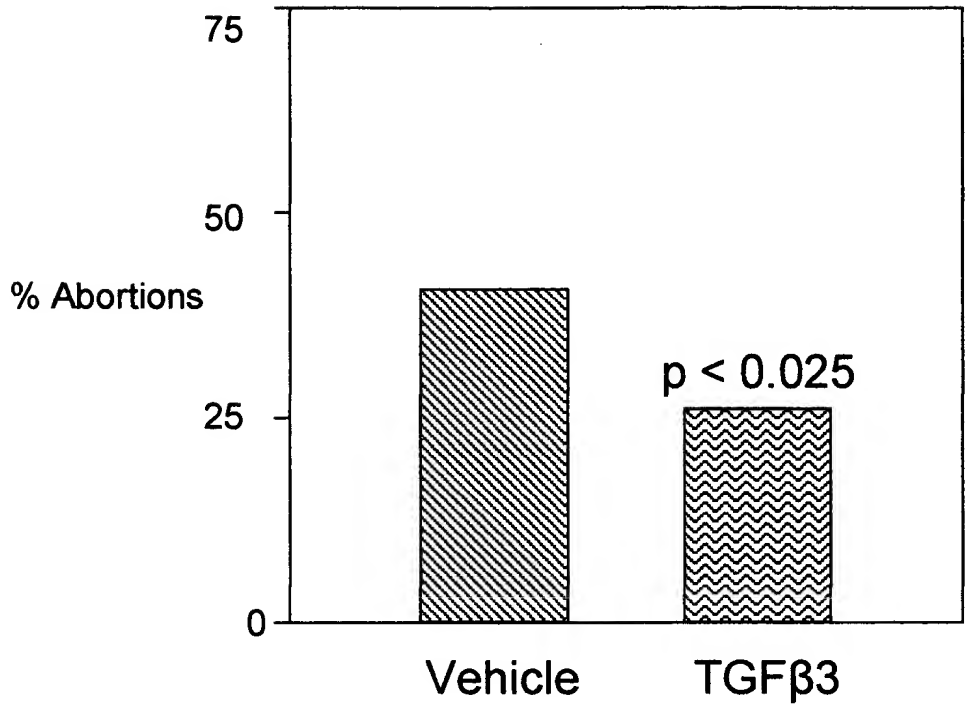
16. Recombinant TGF-β3 was administered vaginally (pv) in this model of recurrent miscarriage to test the ability of TGF-β3 to reverse the high levels of miscarriage by influencing the maternal immune status towards a pro-pregnancy Th2-type response. A single dose of vehicle (10 microlitres of 0.1% Bovine Serum Albumin in phosphate-buffered saline) or TGF-β3 (20 nanograms in 10 microlitres of vehicle) was administered either before or after mating. Following mating, the female mice were treated with an intra-peritoneal (ip) dose of lipopolysaccharide (LPS) (1 microgram per 100 microlitres) to increase the recurrent miscarriage rate. 13.5 days after mating the animals were killed, and the uteri were removed on day 13.5 of gestation for assessment of

(a) the number of mice with embryos (the pregnancy rate), and

(b) the number of implantations and the number of resorptions (the resorption rate).

17. In the vehicle-treated control group 15 animals were mated; of these 11 animals became pregnant, carrying a total of 97 implantations: 41% of these implantations were subsequently lost. In the TGF-β3 treated group 14 animals were mated; all animals became pregnant, producing 115 implantations, of which 26% were subsequently lost. These results are summarized in Figure 1.

FIGURE 1



18. Thus human TGF-β3 significantly reduced the proportion of miscarriage in this CBA x DBA/2 mouse model of recurrent miscarriage. The result is highly statistically significant, as measured by the Chi-squared test, with Yates' correction: $2P < 0.05$, Chi-squared = 4.797.

19. This result shows that a single dose of TGF-β3 administered to a prospective mother lacking immune tolerance to paternal antigen, either before attempted conception or after attempted conception, actually prevents miscarriage and has no detrimental affect on pregnancy. I consider that this result shows that TGF-β3 is interchangeable with TGF-β1 and TGF-β2 in the method of the invention.

20. This result was expected from Table V in the present specification, which shows that treatment with sperm + TGF-β1 increased fetal and placental weight in a BALB/c x CBA mating combination. The results presented above show an increased rate of successful implantation, which was also predictable from Table VI in the present specification.

21. In the experiment described herein, and as disclosed and claimed in the patent specification, TGF-β is administered to the prospective mother pv, ie via a mucosal surface. This mucosal administration, as used in the experiment described herein in accordance with the

teachings and claims of the present specification, proved to be safe and effective, contrary to what would have been expected before 1997.

22. The phenomenon reported by Chaouat et al was not attributable to induction of tolerance, and the results of the experiment reported here indicate that administering TGF-β locally into the vaginal tract one day before or one day after natural introduction of DBA/2 semen reduced the abortion rate. In contrast to the report of Chaouat et al, it was unnecessary to use a third party donor. Prior to the teachings of the present invention, no one to my knowledge had ever before reported being able to induce protection against abortion by using male DBA/2 cells.

23. I also consider that this result confirms that a person who was in possession of the present specification, using methods which were widely available at the priority date of the present application, would be able to identify TGF-β3 as suitable for use in the invention, and that these methods would be a matter of routine for a person of ordinary skill in the art.

24. Finally, this result again confirms what is taught in the specification, that the invention achieves an unexpected beneficial result when compared to what the prior art would have taught at the priority date of the invention.

I declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by prison or fine or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any application or patent thereon.

DECLARED at Burlington this 2 day of July 2004

David A. Clark

David A. Clark

Before me:

July 2, 2004, at Burlington, Ontario

FULVIO DELIBATO

NOTARY PUBLIC (COMMISSION DOES NOT EXPIRE)

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada.

IN THE MATTER OF
US Patent Application No. 09/380,327
by Robertson et al

EXHIBIT DAC-1

This is Exhibit DAC-1 referred to in the Statutory Declaration dated 2 July 2004 by David Alexander Clark.

David A. Clark

Before me:

July 2, 2004, @ Burlington, Ontario.

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada

FULVIO DELIANTO
NOTARY PUBLIC
etc.

BIOLOGY OF REPRODUCTION 46, 561–572 (1992)

Localization of Transforming Growth Factor-β at the Human Fetal-Maternal Interface: Role in Trophoblast Growth and Differentiation[1]

CHARLES H. GRAHAM,[2,3] JEFFREY J. LYSIAK,[3] KEITH R. McCRAE,[4] and PEEYUSH K. LALA[3]

Department of Anatomy,[3] University of Western Ontario, London, Ontario, Canada N6A 5C1
Hematology-Oncology Section,[4] Department of Medicine, School of Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104-4283

ABSTRACT

We examined the localization of transforming growth factor (TGF)-β in first-trimester and term human decidua and chorionic villi and explored the role of this factor on the proliferation and differentiation of cultured trophoblast cells. Two antibodies, 1D11.16.8, a mouse monoclonal neutralizing antibody capable of recognizing both TGF-β1 and TGF-β2 and CL-B1/29, a rabbit polyclonal antibody capable of recognizing TGF-β2, were used to immunolocalize TGF-β in fixed, paraffin-embedded, or fixed, frozen sections of placenta and decidua, providing similar results. Intense labeling was observed in the extracellular matrix (ECM) of the first-trimester decidua and cytoplasm of term decidual cells. Syncytiotrophoblast cell cytoplasm as well as the ECM in the core of the chorionic villi of both first-trimester and term placentas exhibited a moderate degree of labeling. Strong cytoplasmic labeling was observed in the cytotrophoblastic shell of the term placenta. To examine the role of TGF-β on trophoblast proliferation and differentiation, early passage cultures of first-trimester and primary cultures of term trophoblast cells were established and characterized on the basis of numerous immunocytochemical and functional markers. These cells expressed cytokeratin, placental alkaline phosphatase, urokinase-type plasminogen activator, and pregnancy-specific β glycoprotein, but not factor VIII or 63D3; they also produced hCG and collagenase type IV. Exposure of first-trimester trophoblast cultures to TGF-β1 significantly inhibited proliferation in a dose-dependent manner. An antiproliferative effect was also noted in the presence of TGF-β2. These effects were abrogated in the presence of the neutralizing anti-TGF-β antibody (1D11.16.8) in a concentration-dependent manner. In a 3-day culture, exogenous TGF-β1 stimulated formation of multinucleated cells by the first trimester as well as term trophoblast cells. Addition of neutralizing anti-TGF-β antibody to first-trimester trophoblast cells stimulated proliferation beyond control levels in a 24-h culture and reduced formation of multinucleated cells in a 3-day culture, indicating the presence of endogenous TGF-β activity. These results indicate that TGF-β produced at the human fetal-maternal interface plays a major regulatory role in the proliferation and differentiation of the trophoblast.

INTRODUCTION

Transforming growth factor-β (TGF-β) is a family of polypeptides that has the ability to regulate in vitro differentiation and proliferation of a variety of cell types depending on the microenvironment [1]. It has been shown to inhibit proliferation of epithelial cells and to induce their differentiation [2–5]. One of the initial sources from which TGF-β was purified to homogeneity was the human placenta [6]. This organ also has high-affinity receptors for TGF-β [7]. However, the precise cellular source of TGF-β and its functional role at the fetal-maternal interface remain to be fully defined. Recently, we have shown that first-trimester human decidual cells and to some extent trophoblast cells in culture produce TGF-β and that it is instrumental in the control of trophoblast invasion [8–10]. In the present study we examined the localization of TGF-β at the fetal-maternal interface and its possible functional role in trophoblast proliferation and differentiation in vitro.

Human placental trophoblast cells consist of villous trophoblast and extravillous trophoblast. Both villous and extravillous populations include subpopulations of mononucleated (cytotrophoblast) and multinucleated (syncytial) cells. Proliferative ability of trophoblast cells in the chorionic villus as well as extravillous sites is confined to the cytotrophoblast. Multinucleated trophoblast cells in the chorionic villus are designated as syncytiotrophoblast, whereas those in extravillous locations (placental bed) are called placental bed giant cells. Multinucleated trophoblast cells, which are nonproliferative, arise as the result of fusion and differentiation of cytotrophoblast cells in both cases. Little is known about the molecular signals that regulate proliferation of trophoblast cells and their differentiation into multinucleated cells. We demonstrate here that TGF-β provides one such signal.

MATERIALS AND METHODS

Immunohistochemical Staining for TGF-β

Human first-trimester decidual tissue and chorionic villi (resulting from elective terminations of pregnancy) and selected samples from term placentas (obtained after normal delivery) were fixed in Bouin's fixative and embedded in paraffin, or fixed with 0.1% glutaraldehyde/3% paraformaldehyde in PBS and frozen. Immunohistochemical staining for TGF-β was performed using two antibodies (Abs): CL-B1/29, a rabbit polyclonal Ab capable of recognizing TGF-β2 [11], and 1D11.16.8, a mouse monoclonal neutralizing

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[2]Correspondence. FAX: (519) 661-3797.

Ab capable of recognizing both TGF-β1 and TGF-β2 [12]. Both Abs were generously provided by Celtrix Laboratories (Palo Alto, CA). The dilution for the polyclonal Ab was 125 μg/ml, according to the pretested protocol provided by Dr. Howard Higley (Celtrix Laboratories). The dilution of the monoclonal Ab was 8 μg/ml, as determined from pilot studies using serial dilutions. Sections incubated in the absence of primary Ab, in the presence of equivalent dilutions of normal rabbit serum, or in the presence of Ab preabsorbed with purified TGF-β2 served as negative controls for CL-B1/29. Negative controls for 1D11.16.8 consisted of sections treated without the primary Ab or incubated with an unrelated anti-H-2K^k Ab of identical Ig class. Immunostaining was performed by means of the avidin-biotin-peroxidase (ABC) technique with diaminobenzidine (DAB) as the chromogen [13].

To determine the precise identity of certain cells that localized TGF-β, immunohistochemical staining for cytokeratin was performed in serial sections, since cytokeratin provides the most reliable marker for trophoblast cells in situ in the placenta [14]. Sections of term placental tissue were incubated with a monoclonal Ab that recognizes human cytokeratins 8 and 18 (Becton, Dickinson, San Jose, CA). Negative controls were provided by sections treated without primary Ab or incubated with an unrelated anti-H-2K^k Ab of identical Ig isotype. Immunocytochemistry was then performed using the ABC technique with DAB as the chromogen.

Establishment of First-Trimester Trophoblast Cells in Culture

First-trimester human trophoblast cells were cultured as detailed elsewhere [8] using a modification of the method of Yagel et al. [15] previously reported from our laboratory. Briefly, first-trimester placental tissues were rinsed in cold RPMI 1640 medium (Grand Island Biological Company, Grand Island, NY). Chorionic villi were minced finely and washed once in RPMI 1640 medium. Villus fragments were plated in culture medium consisting of RPMI 1640, 10% fetal calf serum (FCS), streptomycin (200 μg/ml), penicillin (200 U/ml), and amphotericin B (0.50 μg/ml) in 75-cm^2 closed tissue culture flasks. Fragments were allowed to adhere for 2–3 days, after which nonadherent cells were removed and discarded. Adherent cells were expanded for 1–2 wk during which the medium was changed every 3 days. For passages, cells were removed by trypsinization (0.25% trypsin in Ca^{2+}- and Mg^{2+}-free PBS + 0.02% EDTA), washed, and plated in similar flasks. For long-term storage, cultures were kept frozen in liquid nitrogen in a mixture of 50% culture medium, 45% fetal calf serum, and 5% dimethylsulfoxide.

Immunohistochemical Characterization of First-Trimester Trophoblast Cells in Culture

First-trimester trophoblast cultures, after 1–5 passages, were characterized morphologically and by immunocytochemical staining using the following panel of Abs: (1) a mouse monoclonal Ab to cytokeratin (Becton, Dickinson) that recognizes human cytokeratin filaments 8 and 18 (negative controls consisted of mouse monoclonal anti-H-2K^k [IgG_{2a}, same isotype as the anti-cytokeratin Ab] applied to similar cultures, cultures processed without primary Ab, and cultured fibroblasts from term placentas); (2) monoclonal Abs against cytokeratins 7 and 18 (Boehringer-Mannheim, FRG); (3) monoclonal Abs A1E5, RC311, and 2C5 (kindly provided by Drs. M. Lafferty and H. Harris, Department of Genetics, University of Pennsylvania, Philadelphia, PA), all against placental alkaline phosphatase; (4) polyclonal rabbit Ab against urokinase-type plasminogen activator (uPA; American Diagnostic, Greenwich, CT); (5) monoclonal Ab against 63D3, a human macrophage marker (hybridoma obtained from the American Type Culture Collection, Rockville, MD [16]; human decidual macrophages served as positive control); and (6) polyclonal rabbit Ab against Factor VIII (Dakopatts, Glostrup, Denmark; sections of rat brain blood vessels were used as positive controls). The ABC technique was used to localize antigens.

Functional Characterization of First-Trimester Trophoblast Cells in Culture

Levels of hCG were measured in the conditioned media of primary and passaged, subconfluent first-trimester trophoblast cultures at the Department of Nuclear Medicine, St. Joseph's Hospital, London, Ontario, by means of a microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL). Briefly, diluted samples of conditioned media were treated with an anti-hCG (recognizing the beta subunit of hCG) Ab conjugated with alkaline phosphatase. This enzyme-Ab-antigen complex was then incubated with anti-hCG-coated microparticles, and an aliquot of this mixture was transferred to a glass fiber matrix. The matrix was then

FIG. 1. a) Photomicrograph of a paraffin section of human first-trimester decidua stained for TGF-β with the monoclonal Ab 1D11.16.8. Note intense labeling of the ECM throughout the decidua and intracellular staining in a minority of decidual cells (arrow). The majority of decidual cells (large cells with eccentric nuclei) and decidual leukocytes are not stained. ×672. b) Photomicrograph of a paraffin section of human first-trimester chorionic villi stained with the monoclonal Ab. Strong cytoplasmic staining is seen in the syncytiotrophoblast (SYN) layer; minor staining is noted in the villus core (VC). Little or no labeling is seen in the cytotrophoblast cell layer (arrows). ×672. c) Paraffin section of human term placenta and associated decidua stained for cytokeratin. Specific staining of the villous syncytiotrophoblast (arrow) and the cells of the cytotrophoblastic shell (CS) is seen. Decidua (D) and the core of the villi (VC) are negative. ×168. d) Paraffin section of human term placenta and associated decidua stained with monoclonal Ab 1D11.16.8. Intense cytoplasmic staining is observed within decidual cells (D) and trophoblast cells of the cytotrophoblastic shell (CS). The syncytiotrophoblast (arrow) and core of the villi (VC) exhibit weak labeling. ×168. e) High magnification of human term decidual cells stained with monoclonal anti-TGF-β Ab. Strong intracellular labeling is seen within decidual cells and an absence of labeling of the ECM. ×672. f) Human term placenta and decidua stained with the monoclonal Ab H-2K^k, used as a negative control. No staining is seen in the decidua (D), cytotrophoblastic shell (CS), or chorionic villi (CV). ×168.

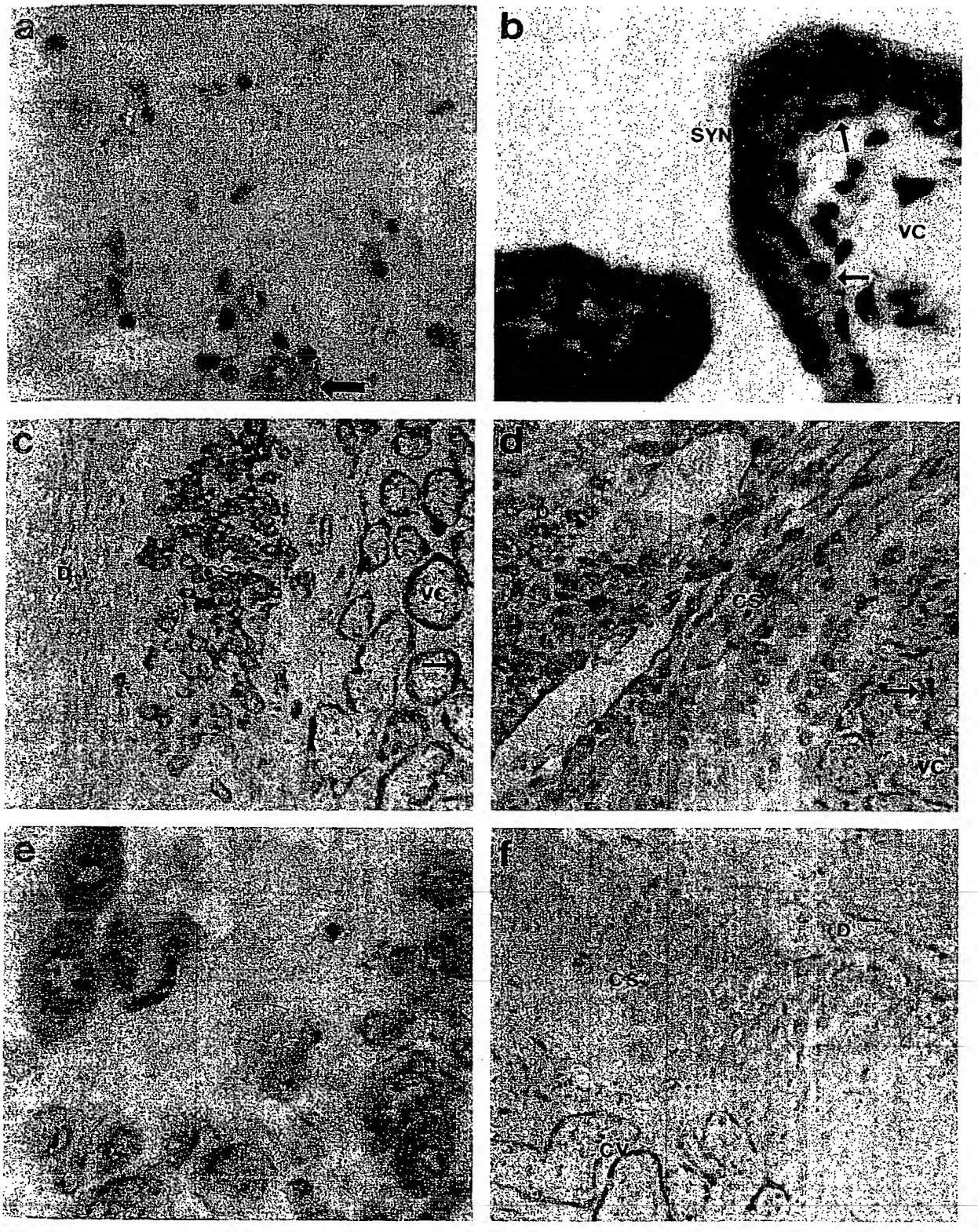

a
b
SYN
VC
c
VC
d
CS
VC
e
f
D
CS
CV

FIG. 2. Morphology of first-trimester trophoblast cells in culture a) A morphologically heterogeneous population remains after 2 passages. b) Multinucleated cell formation takes place even after 11 passages. Phase contrast, ×267.

washed to remove unbound material and the substrate 4-methylumbelliferyl phosphate was added, and the resulting fluorescence was measured. The sensitivity of this assay has been calculated to be better than 2.0 mIU/ml.

Collagenase type IV activity (a characteristic of invasive trophoblast cells) was measured in the conditioned media of first-trimester (fourth-passage) trophoblast cells using the method of Vaithilingam et al. [17]. Briefly, collagenolytic activity was evaluated by measuring breakdown products of ^{3}H-labeled collagen type IV after precipitation with trichloroacetic acid.

Culture and Characterization of Term Trophoblast Cells

Attempts to grow term placental trophoblast cells by culturing term chorionic villus fragments in the same manner as described for first-trimester chorionic villi did not suc-

TABLE 1. Immunocytochemical characteristics of first-trimester trophoblast cells (2–4 Passages).

Marker	% Positive
Cytokeratins 8 and 18	100
Cytokeratin 18	100
Cytokeratin 7	100
Placental alkaline phosphatase:	
A1E5	100
RC311	100
2C5	100
Urokinase-type plasminogen activator	100
63D3 (macrophage marker)	0
Factor VIII	0

ceed. After one or two passages, cultures were overgrown to confluence by fibroblasts, although a few cytokeratin-positive cells remained. These cultures were used as negative controls for immunocytochemical studies. We succeeded in isolating term cytotrophoblast cells for primary cultures by employing the method of Kliman et al. [18]. Briefly, term placentas were washed, and the decidual tissue and vessels were carefully removed. Placental tissue was then minced and subjected to three cycles of digestion with 0.125% trypsin and 0.2 mg/ml DNAase type I. The cells released in each cycle of digestion were pelleted through calf serum, resuspended in Dulbecco's Modified Eagle Medium containing 4.5 g/L glucose (DMEM-HG), and pooled. Aliquots were centrifuged through a 5–70% discontinuous percoll gradient for 30 min at 1 400 × *g*. Trophoblasts were identified as a large band with a density of about 1.050. The isolated trophoblast (primarily cytotrophoblast) population was at least 95% pure as determined by immunocytochemical staining with monoclonal Abs for cytokeratins 7 and 18, α-hCG,β-hCG, pregnancy-specific β_1 glycoprotein (Dakopatts), and placental alkaline phosphatase and by lack of staining with Abs specific for vimentin (Cadna Medical Diagnostic Inc., Toronto, Ontario) and the monoclonal Ab 63D3 (against human macrophages). Term trophoblasts were cultured in DMEM-HG containing 10% FCS, 2 mM glutamine, and 0.05 mg/ml gentamycin. All studies with these cells were performed on primary cultures.

Conditions of Trophoblast Cultures for Examining the Role of TGF-β

Effects on cell proliferation. In the first set of experiments, designed to test TGF-β effects on cell proliferation, 50-μl aliquots containing 5.0 × 10^5 first-trimester trophoblasts after 2–3 passages were added to each well of a 96-well (flat-bottom) microtiter plate (Flow Laboratories, McLean, VA), and allowed to adhere for 24 h. Fifty microliters of various dilutions of porcine TGF-β1 with full biological activity for human cells (R & D Systems, Minneapolis, MN) was then added to each well (for a final concentration range of 0.0001–0.10 ng/ml TGF-β1) and incubated for a further 24 h before harvesting. Six hours before harvesting, 50 μl of tissue culture medium containing 0.15 μCi ^{3}H-thymidine (^{3}H-TdR) was added to each well (for a final concentration of 1 μCi/ml). At the end of the incubation period, the tissue culture medium was removed, and 100 μl of 0.25% trypsin was added to each well. The cells were harvested 15 min later by means of a Titertek harvester, and 2-min counts were taken on a Beckman scintillation counter.

In the second set of experiments, performed to test whether exogenous TGF-β affected cell numbers, first-trimester trophoblast cells were plated in triplicate on 6-well plates (6.0 × 10^4 cells per well; flat-bottom Falcon plates, Becton, Dickinson) and cultured in the absence or presence of TGF-β1 (10 ng/ml). After a 6-day incubation, the cells were lifted from the wells with 0.25% trypsin and counted in a hemocytometer.

In the third set of experiments, designed to examine whether the trophoblast cultures were influenced by endogenous TGF-β activity, first-trimester trophoblast cells were plated in the absence or presence of neutralizing anti-TGF-β monoclonal Ab 1D11.16.8 (25 μg/ml) or TGF-β1 (10 ng/ml). Use of an unrelated monoclonal Ab of the same Ig isotype and in the same concentration (anti-TGF-α; Berlex Bioscience, Alameda, CA) provided an additional internal control. Incorporation of ^{3}H-TdR was determined during the final 6 h of 24-h culture under the above conditions.

In the fourth set of experiments, we tested the effects of exogenous TGF-β1 and TGF-β2 (porcine, R & D Systems; 10 ng/ml) alone or in combination with increasing concentrations (5–100 μg/ml) of neutralizing anti-TGF-β Ab (1D11.16.8) on ^{3}H-TdR uptake by first-trimester trophoblast cells using the same protocol as above. This allowed us to examine the specificity of action of TGF-β and the Ab.

Effects on the formation of multinucleated cells. To examine the effects of TGF-β on the formation of multinucleated cells, 3.0 × 10^4 first-trimester and term trophoblast cells were plated in quadruplicate in 6-well plates and cultured for 72 h in the presence or absence of TGF-β1 (10 ng/ml) or anti-TGF-β (25 μg/ml). Cells were fixed in methanol for 3 min at −20°C and stained with hematoxylin to facilitate counting of nuclei.

TABLE 2. Functional properties of first-trimester trophoblast cells.

hCG production (β subunit)	IU/L
First-trimester trophoblast	
Primary (3-day media)	211
Primary (7-day media)	932
Primary (15-day media)	>4000
Passage I (3-day media)	44
Passage IV (3-day media)	45
Passage V (3-day media)	37
Jar Choriocarcinoma (2-day media)	665
JEG Choriocarcinoma (2-day media)	80
Collagenase Type IV Activity	**U/mg protein**
First-trimester trophoblast	
Passage IV (6-day media)	39, 42

Statistical Analysis

Significance of differences between mean values was evaluated by one-way analysis of variance. A p value ≤ 0.05 was considered significant.

RESULTS

Localization of TGF-β in First-Trimester and Term Decidua and Chorionic Villi

Use of both the monoclonal and the polyclonal anti-TGF-β Abs provided similar patterns of staining. Intense labeling was observed in the extracellular matrix (ECM) of first-trimester decidual tissue; decidual leukocytes and glandular epithelium remained unlabeled (Fig. 1a). First-trimester chorionic villi exhibited strong labeling of syncytiotrophoblast cell cytoplasm as well as moderate labeling of the ECM of the villus core (Fig. 1b). Term placental tissue showed strong intracellular labeling of decidual cells (Figs. 1,d and e) as well as cytotrophoblastic shell embedded in the decidua (Fig. 1d). The latter cell population was distinguished from the decidual cells on the basis of strong staining for cytokeratin (Fig. 1c). Cytokeratin, an intermediate filament of epithelial cells, was localized in cytotrophoblast as well as in syncytiotrophoblast cells. Decidual cells and the mesenchymal core of the chorionic villi were negative for cytokeratin (Fig. 1c). Syncytiotrophoblast cells of the term chorionic villi showed weak-to-moderate labeling for TGF-β (Fig. 1d). A moderate degree of labeling was again observed in the core of the villi (Fig. 1d). No staining was seen in negative control sections in which anti-H-2K^k was used as the primary Ab (Fig. 1f). Omission of the primary Ab or preabsorbion of the polyclonal Ab with purified TGF-β2 abolished the staining (pictures not shown).

Characterization of Cultured First-Trimester Trophoblast Cells

The success rate of growing first-trimester cells with confirmed trophoblastic characteristics ranged between 30% and 50% with the methodology used. Cultures that did not prove to be 100% cytokeratin-positive after one or two passages were discarded. Morphological findings of first-trimester trophoblasts were in general agreement with those of Yagel et al. [15]. After 4–8 days in primary culture, various cell populations could be observed to be radiating from villous fragments. After two passages, a multiangular cell population predominated (Fig. 2a). Signs of formation of multinucleated cells were still evident after eleven passages (Fig. 2b). The cultures used in this study were 100% positive for all the cytokeratins examined, indicating an epithelial (trophoblastic) origin for the cells (Table 1). In addition, 100% of the cells were positive for placental alkaline phosphatase and uPA. None of the cells were found to be positive for factor VIII, or the macrophage marker 63D3, thus excluding the possibility that they may be endothelial cells or cells of the monocyte-macrophage lineage.

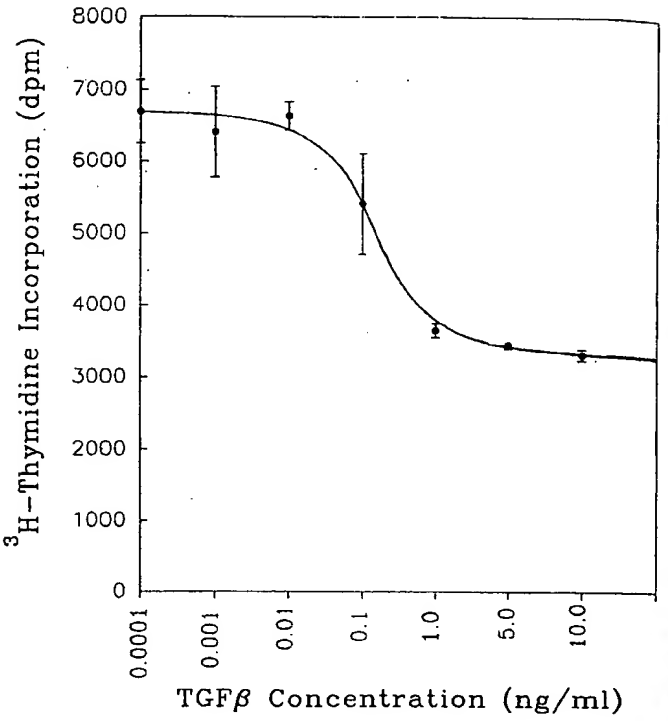


FIG. 3. Effect of various doses of TGF-β1 on trophoblast proliferation (^{3}H-TdR uptake). Each bar represents the mean (±SE) of quadruplicate wells.

Human CG production was maintained in these cultures until at least five passages. However, the levels decreased significantly after passaging of the cells (Table 2). First-trimester trophoblast cell cultures also produced substantial levels of collagenase type IV (Table 2) and plasminogen activator (data not shown). Furthermore, they expressed saturable binding sites for single-chain uPA ([9], McCrae et al., unpublished results). Employing an in vitro invasion assay, we have also shown that these cells are highly invasive [10].

Characterization of Term Trophoblast Cells in Culture

The morphology of isolated term trophoblast cells was as described by Kliman et al. [18]. A large proportion of the cells (38%) after 3 days in culture were multinucleated. This occurred despite the fact that the isolation procedure preferentially isolates cytotrophoblasts. Their marker characteristics have already been listed earlier in the *Materials and Methods* section. Term trophoblast cells also expressed saturable binding sites for uPA, although to a lesser extent than first-trimester cells ([9], McCrae et al., unpublished results).

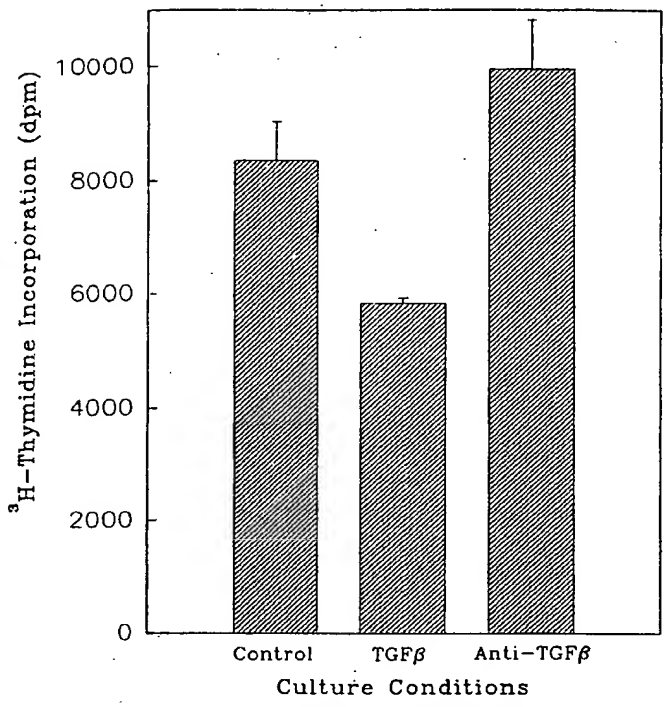


FIG. 4. ^{3}H-TdR uptake by cultured (3 passages) first-trimester trophoblast cells under various conditions: TGF-β = 10 ng/ml; anti-TGF-β = 25 μg/ml.

Effect of TGF-β on First-Trimester Trophoblast Proliferation

Incubation of trophoblast cells in the presence of TGF-β1 for 24 h reduced ^{3}H-TdR incorporation in a dose-dependent manner (Fig. 3). Significant ($p < 0.011$) suppression was observed at doses of 1–10 ng/ml. In accordance with these results, incubation of cells in the presence of TGF-β1 (10 ng/ml) for 6 days resulted in a 35% decline ($p < 0.01$) in the total cell number from (3.25 ± 0.14) × 10^5 cells in the control to (2.11 ± 0.19) × 10^5 cells in the TGF-β1-treated group.

Addition of anti-TGF-β Ab in a 24-h culture resulted in a small ($p = 0.2$) increase in ^{3}H-TdR incorporation over control cultures (Fig. 4), indicating the possible presence of endogenous TGF-β. Addition of another Ab (anti-TGF-α) of the same isotype and in the same concentration as the anti-TGF-β Ab did not affect ^{3}H-TdR uptake as compared with cells cultured in medium alone (not shown).

In another experiment (Fig. 5), exogenous TGF-β1 as well as TGF-β2 (10 ng/ml) reduced ^{3}H-TdR uptake by trophoblast cells. Additional presence of the neutralizing Ab abrogated these effects in a dose-dependent manner. The abrogation of TGF-β1 activity was complete and significant ($p = 0.002$) at an Ab concentration of 25 μg/ml; for TGF-β2 the effective concentration was 50 μg/ml ($p = 0.01$). The Ab alone caused a minor dose-dependent stimulation of ^{3}H-TdR uptake. Taken together, these results indicate that the anti-proliferative function of TGF-β is specific.

Effects of TGF-β1 and Neutralizing Anti-TGF-β Ab on Formation of Multinucleated Cells by First-Trimester and Term Trophoblast Cells

Figures 6 and 7 show that the presence of exogenous TGF-β1 (10 ng/ml) in 3-day cultures of first-trimester and term trophoblasts induced formation of multinucleated cells. We defined multinucleated cells as cells having two or more nuclei. Results in these figures are given as the percentage of the total number of cells counted; they indicate that addition of TGF-β1 to both first-trimester and term trophoblast cultures caused a shift in the ratio of mononucleated to multinucleated cells, the latter becoming the predominant cell type ($p < 0.003$ for first-trimester trophoblast and $p < 0.0003$ for term trophoblast). Addition of anti-TGF-β Ab to first-trimester trophoblasts significantly reduced the number of multinucleated cells. This reduction in multinucleated cell formation by the Ab was evident when the Ab-containing medium was compared with both the me-

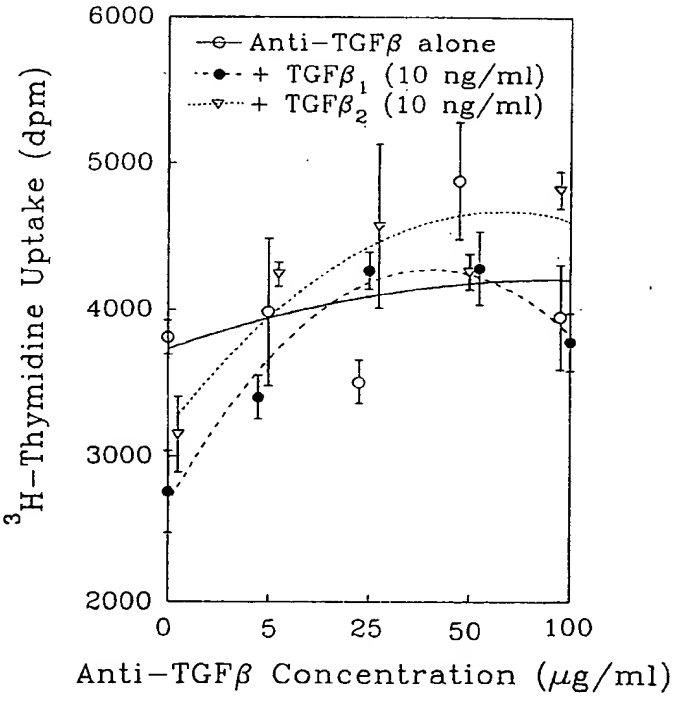


FIG. 5. Effect of various concentrations of neutralizing monoclonal anti-TGF-β Ab on ^{3}H-TdR incorporation by first-trimester trophoblast cells cultured in the presence or absence of TGF-β1 or TGF-β2 (10 ng/ml). Note that presence of both TGF-β molecules in the absence of Ab resulted in decreased ^{3}H-TdR uptake and that addition of Ab in increasing concentrations resulted in a gradual increase and restoration of ^{3}H-TdR uptake. Addition of Ab alone resulted in a minor (nonsignificant) dose-dependent increase in uptake. Symbols represent the mean (±SE) of quadruplicate wells. Second-order regression curves were generated with a Sigma Plot Scientific Graph System (Jandel Scientific, Corte Madera, CA).

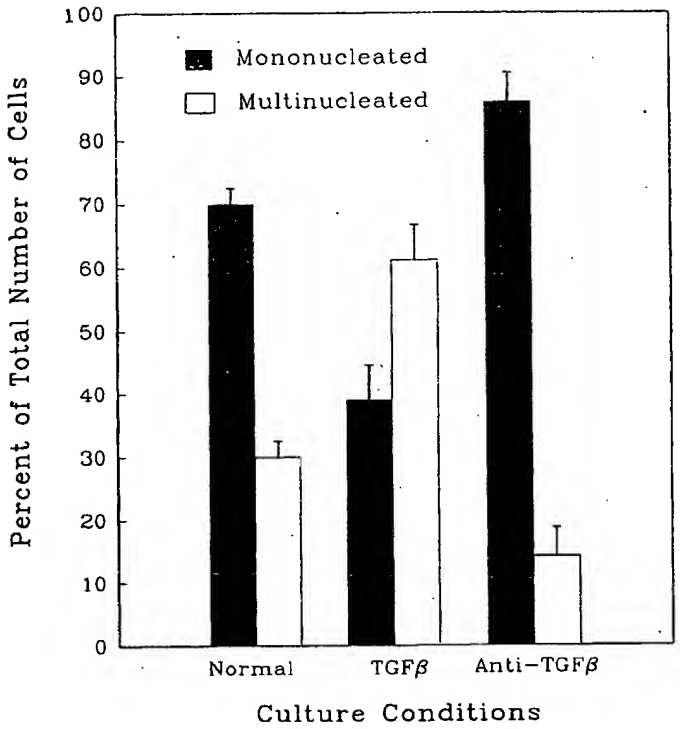


FIG. 6. Effect of TGF-β1 on formation of multinucleated cells by first-trimester trophoblast cells. Cells were counted in quadruplicate wells. Bars represent mean (±SE) percentage of the total number of cells counted in each culture condition. At least 2 000 cells were counted in each group. A cell containing 2 or more nuclei was considered to be multinucleated.

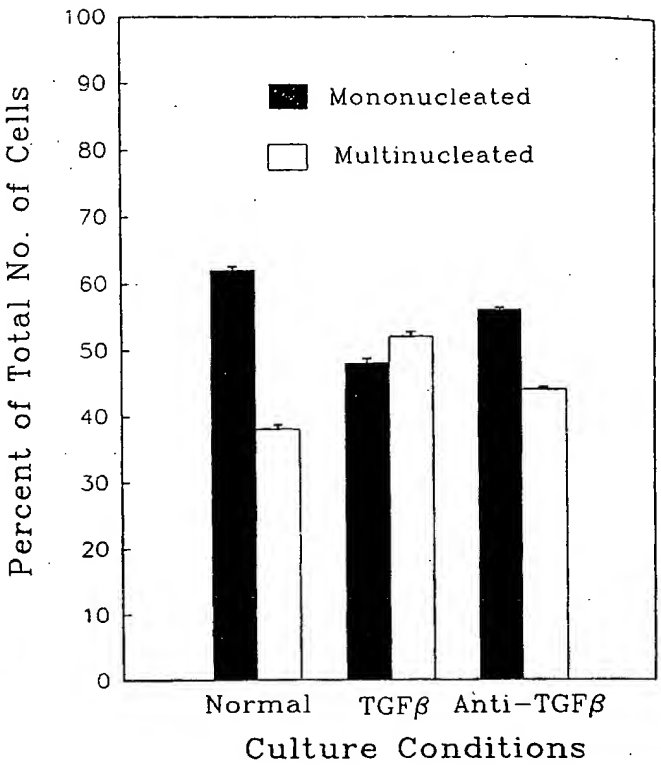


FIG. 7. Effect of TGF-β1 on formation of multinucleated cells by term trophoblast cells. Cells were counted in quadruplicate wells. Bars represent mean (±SE) percentage of the total number of cells counted in each culture condition. At least 2 000 cells were counted in each group.

dium containing exogenous TGF-β ($p < 0.001$) and the control medium ($p < 0.03$). This suggests that either trophoblasts themselves produced TGF-β or that the tissue culture medium contained functional levels of TGF-β. We have earlier shown that first-trimester trophoblast cells do indeed produce bioactive TGF-β [10].

Figure 8 illustrates morphological characteristics of first-trimester trophoblast cells exposed to culture medium alone (Fig. 8a), to TGF-β1 (Fig. 8b), and to anti-TGF-β (Fig. 8c) and then stained immunocytochemically for cytokeratins 8 and 18. One hundred percent of the cells were labeled for cytokeratin under all conditions. When a similar but unrelated Ab was used to replace the anti-cytokeratin Ab, none of the cells showed staining. Furthermore, term placental fibroblasts were negative for cytokeratin (Fig. 8d), indicating that the monoclonal Ab to cytokeratin was specific and effective.

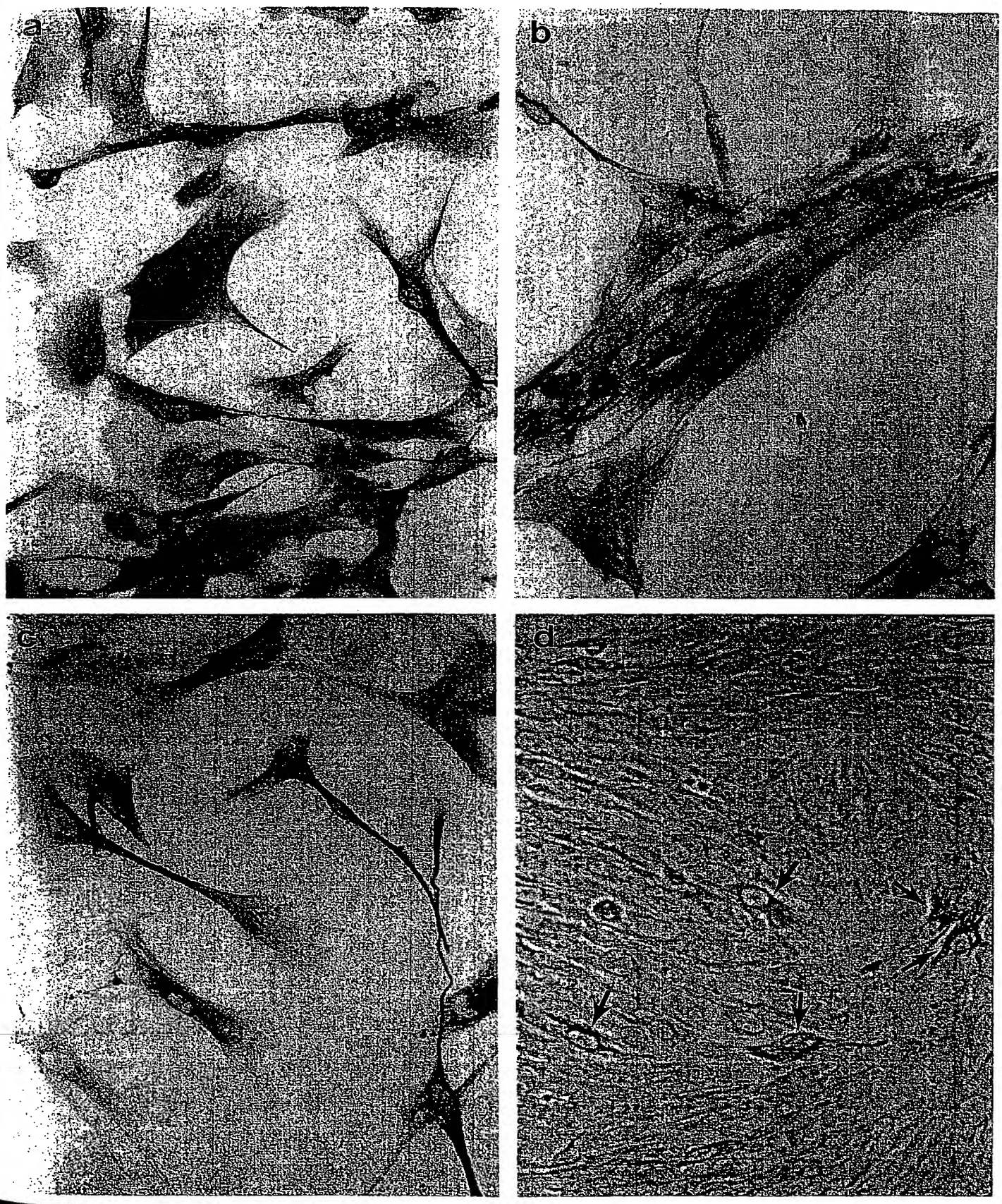
Further analysis of these cultures indicated that addition of exogenous TGF-β1 resulted in an increase in the number of nuclei in the cells. As shown in Figure 9, a large proportion of first-trimester trophoblast cells exposed to exogenous TGF-β1 had five or more nuclei (28.2%), whereas only 3.6% of cells exposed to anti-TGF-β Ab had five or more nuclei.

Similar results were obtained when term trophoblasts were cultured under the above conditions (Fig. 10). In all the above experiments, cell viability at the end of the incubation times was greater than 99%.

DISCUSSION

The present study was designed to identify the location of the TGF-β protein in tissues of the human fetal-maternal interface and to examine the function of this molecule on trophoblast growth and differentiation, employing well-characterized human trophoblast cells in culture.

FIG. 8. Immunolabeling for cytokeratin of first-trimester cells (3 passages) exposed to different culture conditions (a-c, bright-field) and second-passage term placental cultures (d, phase-contrast to show nuclei of unstained fibroblast). All cells in the three conditions were cytokeratin-positive. a) Some degree of multinucleated cell formation was observed in cultures in medium alone. b) A large proportion of cells in cultures treated with TGF-β are multinucleated. c) Cultures treated with anti-TGF-β Ab show a significant reduction in the number of multinucleated cells. d) Second-passage term placental cultures are overgrown by cytokeratin-negative fibroblasts, and only a few cytokeratin-positive trophoblast cells (arrows) remain. All photographs ×256.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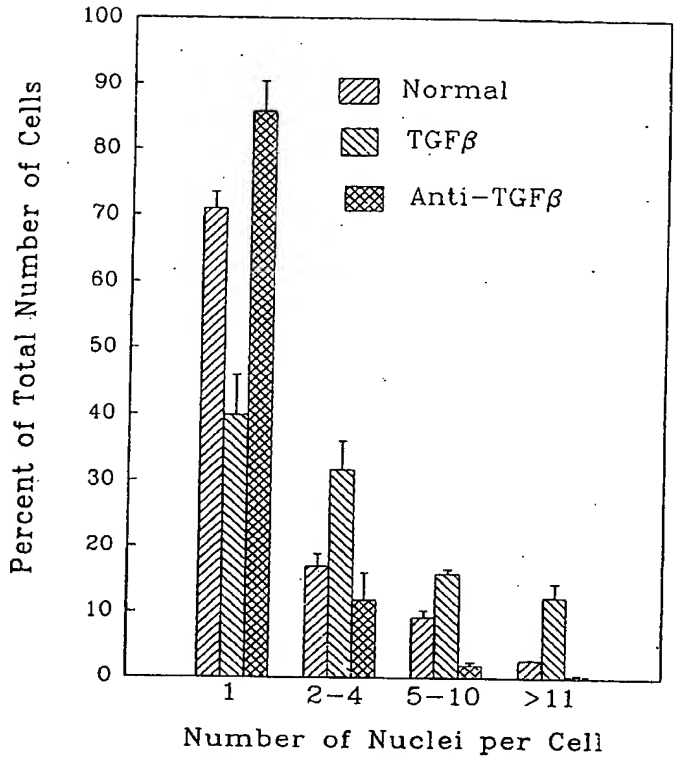

FIG. 9. Effect of TGF-β1 on the number of nuclei in first-trimester trophoblast cells. Bars represent the percentages of the total number of cells counted (±SE). Counts were performed in quadruplicate wells, and at least one thousand cells were counted in each culture condition. TGF-β1 = 10 ng/ml; anti-TGF-β = 25 μg/ml.

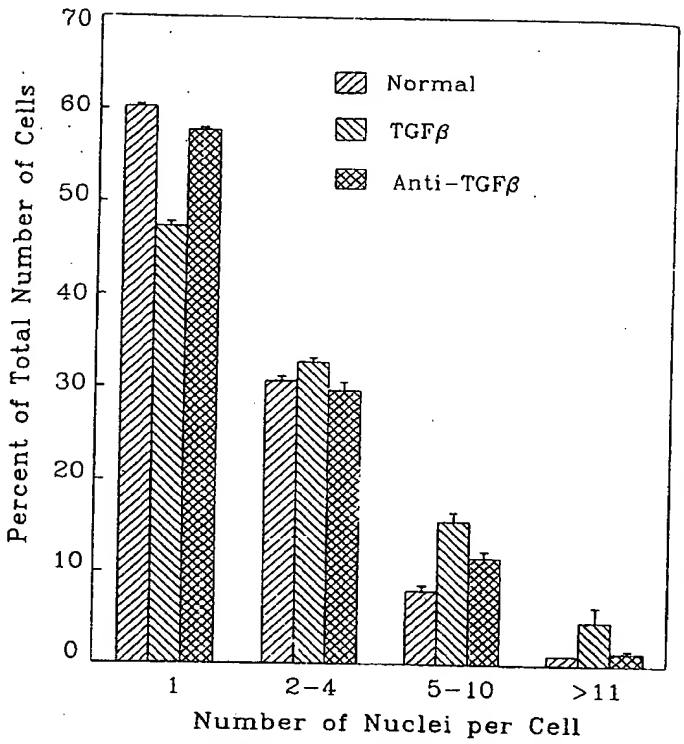

FIG. 10. Effect of TGF-β1 on the number of nuclei in term trophoblast cells. Bars represent the percentages of the total number of cells counted (±SE). Counts were performed in quadruplicate wells, and at least one thousand cells were counted in each culture condition. TGF-β1 = 10 ng/ml; anti-TGF-β = 25 μg/ml.

For immunolocalization, we utilized two different Abs: recognizing both TGF-β1 and TGF-β2, and the other recognizing TGF-β2 alone, both providing similar staining patterns. While these results conclusively demonstrate the presence of TGF-β2, they do not exclude the presence of other forms of TGF-β (β1 or β3). Expression of TGF-β1 has been reported for the mouse peri-implantation uterus [19], whereas TGF-β2 but not β1 mRNA has been reported for the mouse pregnant uterus [20]. In the decidua of the first-trimester, TGF-β was mostly localized in the ECM, whereas in the full-term decidua, it was primarily in the decidual cell cytoplasm. This difference can be due to a higher rate of synthesis and/or a slower rate of release of TGF-β by term decidual cells, as well as a relative paucity of ECM in the term decidua. Decidual ECM may serve as a storage site for TGF-β as has been documented for other tissues [21]. Although immunostaining cannot distinguish between the active and latent forms of TGF-β, we have earlier noted that TGF-β released in the culture medium of the first-trimester decidua is mostly in its latent form [10]. The present study also revealed the presence of TGF-β in the cytoplasm of syncytiotrophoblast cells of the chorionic villi of both first-trimester and term placentas; minor labeling was noted in the ECM of the villus core. The cytoplasm of the extravillous trophoblasts in the cytotrophoblastic shell was also labeled. These observations are consistent with our earlier results showing that a bioactive form of TGF-β is released by trophoblast cells in culture [10]. It is highly likely that decidua-derived TGF-β at the chorio-decidual interface is activated by trophoblast-derived proteinases [9].

Trophoblast cultures established using the methodology described here have been characterized previously [15]. Since establishment of pure human trophoblast cells in culture has been fraught with major difficulties [22] and has been the subject of skepticism, we recognized the importance of further characterization of the cultures employed in the study of TGF-β function. On the basis of numerous immunocytochemical and functional markers expressed by these cells (listed in Tables 1 and 2), they were regarded as 100% pure trophoblast. Whenever cells were positive for cytokeratin, they were also positive for other trophoblast markers. Therefore, cytokeratin is a reliable marker for identifying trophoblast cells in the present system. This conclusion is supported by the in situ immunocytochemical data presented here, as well as those reported by Daya and Sabet [14], in which cytokeratin is shown to be expressed exclu-

sively by trophoblast cells in the placenta. Furthermore, their ability to produce proteinases ([9], present study) as well as their invasive ability in vitro [9, 10, 23] reveals that the culture system used in the study reported here selects for invasive trophoblast cells.

We have demonstrated in this study that TGF-β provides an antiproliferative signal for the first-trimester trophoblast and induces formation of multinucleated cells in vitro by both first-trimester and term trophoblast cells. As term trophoblast cells did not divide, multinucleated cell formation was not necessarily linked to the anti-proliferative function of TGF-β. Since TGF-β is abundant at the fetal-maternal interface, we propose that both decidua-derived as well as trophoblast-derived TGF-β plays an essential role for in vivo differentiation of trophoblast. Whether the molecule responsible for the in vivo differentiation of trophoblasts is TGF-β1 or TGF-β2 remains undetermined because the Ab used in the functional studies neutralizes both classes of TGF-β. The same uncertainty also applies to the antiproliferative effect of TGF-β, since both TGF-β1 and TGF-β2 caused a reduction in proliferation that could be prevented by addition of neutralizing Ab. However, the presence of TGF-β2 (determined by immunohistochemistry) in chorionic villi and decidua in situ suggests an in vivo role for this molecule in trophoblast proliferation and differentiation. While the role of TGF-β on the induction of a functional marker for trophoblast differentiation remains to be tested, it appears that TGF-β induces trophoblasts to leave the cell cycle and form multinucleated cells. These multinucleated cells, resulting from the fusion of invasive cytotrophoblasts, possibly represent the in vivo equivalents of placental bed giant cells rather than the villous syncytiotrophoblast. We noted that both first-trimester and term cytotrophoblast cells isolated by the methods described in the present study are highly invasive [8].

It has been shown that a number of other epithelial tissues respond to TGF-β by concomitant growth inhibition and differentiation [3–5] and that TGF-β interferes with the action of specific mitogens such as epidermal growth factor, TGF-α and platelet-derived growth factor [1]. We have preliminary evidence to suggest that TGF-α antagonizes TGF-β action on trophoblast cell proliferation [24].

In addition to the syncytium-inducing and anti-proliferative function, we have recently shown that TGF-β also plays an important role in controlling trophoblast invasion in situ [8–10]. Using an amnion invasion assay, we showed that conditioned medium from first-trimester decidual cultures suppresses trophoblast invasion. This suppression was relieved by the addition of neutralizing anti-TGF-β Ab and was simulated by TGF-β1. The anti-invasive effect was found to be mediated by the induction of tissue inhibitor of metalloproteinases. Furthermore, it has been suggested that TGF-β2-like molecules secreted by the murine decidua play a local immunoregulatory role [25]. Thus, TGF-β appears to be an important multifunctional molecule at the fetal-maternal interface. The mechanisms that regulate TGF-β production in situ remain to be identified.

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We thank Nahla Al-Mutter for growing term placental fibroblasts used as negative controls and Lawrence Schembri for performing cell counts. We are grateful to Dr. Howard Higley (Celtrix Laboratories, Palo Alto, CA) for his interest and advice on the immunocytochemical component of the study.

REFERENCES

1. Roberts AB, Sporn MB. Transforming growth factor β. Adv Cancer Res 1988; 51:107–145.
2. Like B, Massagué J. The antiproliferative effect of type β transforming growth factor occurs at a level distal from receptors for growth-activating factors. J Biol Chem 1986; 261:12426–12429.
3. Masui T, Wakefield LM, Lechner JF, LaVeck MA, Sporn MB, Harris CC. Type β transforming growth factor is the primary differentiation-inducing serum factor for normal bronchial epithelial cells. Proc Natl Acad Sci USA 1986; 83:2438–2442.
4. Shipley GD, Pittelkow MR, Wille JJ, Scott RE, Moses HL. Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. Cancer Res 1986; 46:2068–2071.
5. Kurokowa M, Lynch K, Podolsky DK. Effects of growth factors on an intestinal epithelial cell line: transforming growth factor β inhibits proliferation and stimulates differentiation. Biochem Biophys Res Comm 1987; 142:775–782.
6. Frolick CA, Dart LL, Meyers CA, Smith DM, Sporn MB. Purification and initial characterization of a type β transforming growth factor from human placenta. Proc Natl Acad Sci USA 1983; 80:3676–3680.
7. Mitchell EJ, O'Connor-McCourt MD. A transforming growth factor β (TGF-β) receptor from human placenta exhibits a greater affinity for TGF-β2 than for TGF-β1. Biochem 1991; 30:4350–4356.
8. Graham CH, Lala PK. Molecular mechanisms controlling trophoblast invasion of the uterus. Trophoblast Res 1992; 7:(in press).
9. Lala PK, Graham CH. Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. Cancer Metastasis Rev 1990; 9:369–379.
10. Graham CH, Lala PK. Mechanism of control of trophoblast invasion in situ. J Cell Physiol 1991; 148:228–234.
11. Ksander GA, Gerhardt CO, Dasch JR, Ellingsworth LR. A novel polyclonal antibody (CL-B1/29) for immunolocalization of transforming growth factor-β2 (TGF-β2) in adult mouse. J Histochem Cytochem 1990; 38:1831–1840.
12. Dasch JR, Pace DR, Waegell W, Inenaga D, Ellingsworth L. Monoclonal antibodies recognizing transforming growth factor β. Bioactivity neutralization and transforming growth factor β2 affinity purification. J Immunol 1989; 142:1536–1541.
13. Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase (ABC) complex in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29:577–580.
14. Daya D, Sabet L. The use of cytokeratin as a sensitive and reliable marker for trophoblastic tissue. Am J Clin Pathol 1991; 95:137–141.
15. Yagel S, Casper RF, Powel W, Parhar RS, Lala PK. Characterization of pure human first-trimester cytotrophoblast cells in long term culture: growth pattern, markers, and hormone production. Am J Obstet Gynecol 1989; 160:938–945.
16. Ugolini V, Nunez G, Smith RG, Stastny P, Capra JD. Initial characterization of monoclonal antibodies against human monocytes. Proc Natl Acad Sci USA 1980; 77:6764–6768.
17. Vaithilingam IS, McDonald W, Stroude EC, Del Maestro RF. General protease and collagenase (IV) activity in C6 astrocytoma cells, C6 spheroids and implanted C6 spheroids. J Neurooncol 1991; 10:203–212.
18. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF. Purification, characterization and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986; 118:1567–1582.
19. Tamada H, McMaster MT, Flanders KC, Andrews GK, Dey SK. Cell type-specific expression of transforming growth factor-β1 in the mouse uterus during the periimplantation period. Mol Endocrinol 1990; 4:965–972.
20. Altman DJ, Schneider SL, Thompson DA, Cheng H, Tomasi TB. A transforming growth factor β2 (TGF-β2)-like immunosuppressive factor in amniotic fluid and localization of TGF-β2 mRNA in the pregnant uterus. J Exp Med 1990; 172:1391–1401.
21. Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB. Expression of transforming growth factor-β1 in specific cells and tissues of adult and neonatal mice. J Cell Biol 1989; 108:661–669.

22. Loke YW. Experimenting with human extravillous trophoblast: a personal view. Am J Reprod Immunol Microbiol 1990; 24:22–28.
23. Yagel S, Parhar RS, Jeffrey JJ, Lala PK. Normal nonmetastatic human trophoblast cells share in vitro invasive properties of malignant cells. J Cell Physiol 1988; 136:455–462.
24. Lala PK, Bonvissuto AC, Graham CH, Lysiak JJ. Localization and function of transforming growth factor α at the fetomaternal interface during first trimester of human pregnancy. Placenta 1991; 12:410–411 (abstr.).
25. Clark DA, Flanders KC, Banwatt D, Millar-Book W, Manuel J, Stèdronska-Clark J, Rowley B. Murine pregnancy decidua produces a unique immunosuppressive molecule related to transforming growth factor β-2. J Immunol 1990; 144:3008–3014.

IN THE MATTER OF
US Patent Application No. 09/380,327
by Robertson et al

EXHIBIT DAC-2

This is Exhibit DAC-2 referred to in the Statutory Declaration dated 2004 by David Alexander Clark.

Before me:

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada

IN THE MATTER OF
US Patent Application No. 09/380,327
by Robertson et al

EXHIBIT DAC-2

This is Exhibit DAC-2 referred to in the Statutory Declaration dated 2 July 2004 by David Alexander Clark.

David A. Clark

Before me: July 2, 2004 @ Burlington, Ontario

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada

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British Journal of Obstetrics and Gynaecology
July 1987, Vol. 94, pp. 649–655

Defective haemochorial placentation as a cause of miscarriage: a preliminary study

T. Y. KHONG, H. S. LIDDELL, W. B. ROBERTSON

Summary. The morphology of the placental bed in idiopathic sporadic and recurrent miscarriages was studied and the findings correlated with the fetal chromosomal pattern where possible. Defective development of haemochorial placentation, which was not necessarily linked with fetal chromosomal abnormality, was seen in association with some miscarriages. These preliminary results, not previously demonstrated, strongly support the concept that miscarriages and pregnancies complicated by pre-eclampsia and/or small-for-gestational-age infants may be a continuum of disorders with a similar pathology in the placental bed.

Many factors are likely to be responsible for miscarriages, most of which are non-recurrent, and although chromosomal abnormalities are one of the most important, their reported occurrence in miscarriages is only about 50% (Boue *et al.* 1985). Furthermore, there is the occasional fetus with a chromosomal abnormality which is not miscarried but attains viability. Causes must be sought for the remaining 50% or more of miscarriages unassociated with chromosomal defects.

It has been suggested that defective placentation may be of crucial import in miscarriage (Robertson 1976; Robertson *et al.* 1985), but this idea has never been fully investigated. Haemochorial placentation is established in the placental bed where the two genetically dissimilar maternal and fetal tissues intermingle intimately. If defective placentation is an important factor underlying miscarriage, it would be expected to be found more frequently in those woman with a history of unexplained recurrent miscarriages. We therefore report what we believe is the first study of the pathology of the placental bed in recurrent miscarriages and in idiopathic sporadic miscarriages with correlation, where possible, with cytogenetic studies of the fetal tissues.

Department of Histopathology, St George's Hospital Medical School, Cranmer Terrace, London
T. Y. KHONG
W. B. ROBERTSON
Department of Obstetrics and Gynaecology, St Mary's Hospital Medical School, London
H. S. LIDDELL

Correspondence: T. Y. Khong, Department of Pathology, John Radcliffe Maternity Hospital, Oxford OX3 9DU

Materials and methods

Materials

Tissue from the placental bed was collected from two centres. Pregnancies of patients with a known history of recurrent miscarriage were monitored by weekly ultrasound scanning and human chorionic gonadotrophin (hCG) assays to assess the viability of their current pregnancies. When either an anembryonic pregnancy or an intrauterine death was diagnosed, these pregnancies were terminated using appropriate methods and placental bed tissue was obtained by curettage immediately before the termination. Under anaesthetic and with the patient's bladder full, an ATL Mk100 sector scanner was used to locate the decidua basalis. A small curette was guided under ultrasound control to the site where the placenta appeared to be localizing and several strips of tissue were taken to try to include the placental bed. This technique was used when the pregnancy was intact.

Table 1. Clinical details of patients and summary of results

Patient no.	Gravidity	Previous pregnancies	Gestational age (weeks)	Fetal chromosome	Uteroplacental circulation	Current pregnancy and comments
Comparison group						
1	1	—	9	46XY	D+	Induced abortion
2	1	—	8	ND	D+	Induced abortion
3	1	—	9	ND	D+	Induced abortion
4	7	SbMLMMM	16	46XX	D+M+	Miscarriage; live fetus; cervical incompetence
Study group						
5	1	—	9	46XX	?*	Missed miscarriage (anembryonic), sac still growing; scarce interstitial trophoblast
6	1	—	13	trisomy 10	D+	Missed miscarriage (anembryonic)
7	1	—	19	ND	D−M−	Missed miscarriage
8	3	LL	12	47XX+22	D+	Missed miscarriage (anembryonic); pregnancy with new partner
9	3	SbM	25	ND	D−M−	Missed miscarriage complicated by pre-eclampsia
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D+/D−, Presence or absence, respectively, of physiological changes in decidual segments; M+/M−, presence or absence, respectively, of physiological changes in myometrial segments.
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Study group. There were seven missed miscarriages in the first trimester, two missed miscarriages and three miscarriages in the second trimester. In two first-trimester and all five second-trimester miscarriages, the uteroplacental vasculature was inadequately developed in that some spiral arteries in the placental bed showed preservation of musculo-elastic tissue with no evidence of involvement with migratory extravillous trophoblast in the decidual segments in the first trimester miscarriages, and in the decidual and myometrial segments in second trimester miscarriages (Figs 3 and 4). Four of the other five first-trimester miscarriages had normally developed physiological changes in the decidual segments of the placental bed spiral arteries. In the remaining case no maternal spiral arteries were seen for assessment of the development of the uteroplacental vasculature but there was a paucity of migratory interstitial trophoblast in the decidua despite the presence of plentiful anchoring villi (Fig. 5). One woman (no. 9) had two earlier pregnancies that were complicated by pre-eclampsia and intrauterine death in the late second trimester. In her current pregnancy, acute atherotic lesions were seen in decidual segments of the spiral arteries (Fig. 6). Acute atherosis was not seen in

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Chromosomal analysis was successful in nine of the 10 attempts. There were three chromosomal abnormalities, one of which was associated with inadequate placentation. There was no correlation between the normality or abnormality of the chromosomal pattern and the normal development or otherwise of the uteroplacental vasculature. Although the numbers are too small for statistical analysis, it would appear that an abnormal uteroplacental vasculature is no more likely to be associated with chromosomally

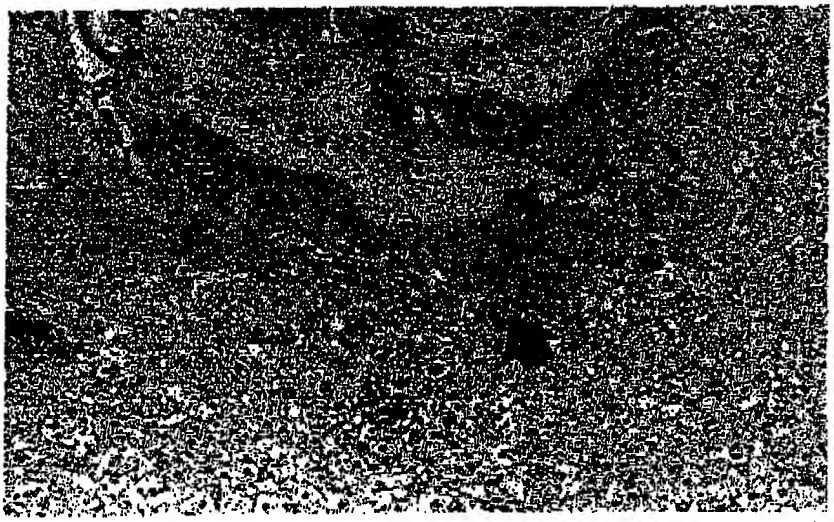

Fig. 5. Placental bed biopsy from missed miscarriage at 10 weeks gestation (patient 5): there is a paucity of migratory interstitial trophoblast in the decidua despite the presence of anchoring villi with a thick rim of cytotrophoblast (arrow). HE × 120.

Fig. 6. Missed miscarriage at 25 weeks gestation which was complicated by pre-eclampsia (patient 9): acute atherosis is seen in a decidual artery. HE × 190.

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These vascular adaptive changes were not observed in two of the seven first-trimester and in none of the five second-trimester miscarriages in the study group, taking into consideration the expected vascular changes at the corresponding gestational ages when these miscarriages occurred. The argument that these adaptive changes would be present had the pregnancies proceeded longer is not tenable as the abnormal vasculature differed too much temporally and topographically from the normal. Nor is it valid to argue that there is no further development of the uteroplacental vasculature after death of the fetus because there is no longer a need for an increasing blood supply; the fetuses in three of the second trimester miscarriages were alive up to the time of the miscarriage, yet with inadequate maternal vascular adaptation.

The only apparent exceptions to the finding of an inadequate maternal vascular response to placentation were in four of the five missed miscarriages without apparent fetuses, the so-called anembryonic conceptuses. There are two possible explanations for this finding. First, such pregnancies if left alone would eventually be expelled from the uterus or, in a proportion of cases, evolve into molar or pseudomolar pregnancies and it is possible that had these four pregnancies continued into the second trimester that transformation of the myometrial segments of the uteroplacental arteries would not have occurred. Second, it is possible that only part of the developing placental bed is abnormally affected and, because only biopsy material was studied, 'false normal' findings are more likely than 'false abnormals' but this would be difficult to validate without hysterectomy specimens.

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References

Boue, A., Boue, J. & Gropp, A. (1985) Cytogenetics of pregnancy wastage. In *Advances in Human Genetics* Vol 14 (Harris, H. & Hirschhorn, K., eds), Plenum Press, New York, pp. 1-57.

Brosens, I., Robertson, W. B. & Dixon, H. G. (1972) The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu* **1**, 177-191.

Khong, T. Y., De Wolf, F., Robertson, W. B. & Brosens, I. (1986) Inadequate maternal vascular response in pregnancies complicated by pre-eclampsia and by small-for-gestational-age infants. *Br J Obstet Gynaecol* **93**, 1049-1059.

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Pijnenborg, R., Bland, J. M., Robertson, W. B., Dixon, G. & Brosens, I. (1981) The pattern of interstitial trophoblast invasion of the myometrium in early human pregnancy. *Placenta* **2**, 303-316.

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Robertson, W. B., Brosens, I. & Landells, W. N. (1985) Abnormal placentation. *Obstet Gynecol Annu* **14**, 411-426.

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Defective haemochorial placentation as a cause of miscarriage: a preliminary study

T. Y. KHONG, H. S. LIDDELL, W. B. ROBERTSON

Summary. The morphology of the placental bed in idiopathic sporadic and recurrent miscarriages was studied and the findings correlated with the fetal chromosomal pattern where possible. Defective development of haemochorial placentation, which was not necessarily linked with fetal chromosomal abnormality, was seen in association with some miscarriages. These preliminary results, not previously demonstrated, strongly support the concept that miscarriages and pregnancies complicated by pre-eclampsia and/or small-for-gestational-age infants may be a continuum of disorders with a similar pathology in the placental bed.

Many factors are likely to be responsible for miscarriages, most of which are non-recurrent, and although chromosomal abnormalities are one of the most important, their reported occurrence in miscarriages is only about 50% (Boue *et al.* 1985). Furthermore, there is the occasional fetus with a chromosomal abnormality which is not miscarried but attains viability. Causes must be sought for the remaining 50% or more of miscarriages unassociated with chromosomal defects.

It has been suggested that defective placentation may be of crucial import in miscarriage (Robertson 1976; Robertson *et al.* 1985), but this idea has never been fully investigated. Haemochorial placentation is established in the placental bed where the two genetically dissimilar maternal and fetal tissues intermingle intimately. If defective placentation is an important factor underlying miscarriage, it would be expected to be found more frequently in those woman with a history of unexplained recurrent miscarriages. We therefore report what we believe is the first study of the pathology of the placental bed in recurrent miscarriages and in idiopathic sporadic miscarriages with correlation, where possible, with cytogenetic studies of the fetal tissues.

Department of Histopathology, St George's Hospital Medical School, Cranmer Terrace, London
T. Y. KHONG
W. B. ROBERTSON
Department of Obstetrics and Gynaecology, St Mary's Hospital Medical School, London
H. S. LIDDELL

Correspondence: T. Y. Khong, Department of Pathology, John Radcliffe Maternity Hospital, Oxford OX3 9DU

Materials and methods

Materials

Tissue from the placental bed was collected from two centres. Pregnancies of patients with a known history of recurrent miscarriage were monitored by weekly ultrasound scanning and human chorionic gonadotrophin (hCG) assays to assess the viability of their current pregnancies. When either an anembryonic pregnancy or an intrauterine death was diagnosed, these pregnancies were terminated using appropriate methods and placental bed tissue was obtained by curettage immediately before the termination. Under anaesthetic and with the patient's bladder full, an ATL Mk100 sector scanner was used to locate the decidua basalis. A small curette was guided under ultrasound control to the site where the placenta appeared to be localizing and several strips of tissue were taken to try to include the placental bed. This technique was used when the pregnancy was intact.

Table 1. Clinical details of patients and summary of results

Patient no.	Gravidity	Previous pregnancies	Gestational age (weeks)	Fetal chromosome	Uteroplacental circulation	Current pregnancy and comments
Comparison group						
1	1	—	9	46XY	D+	Induced abortion
2	1	—	8	ND	D+	Induced abortion
3	1	—	9	ND	D+	Induced abortion
4	7	SbMLMMM	16	46XX	D+M+	Miscarriage; live fetus; cervical incompetence
Study group						
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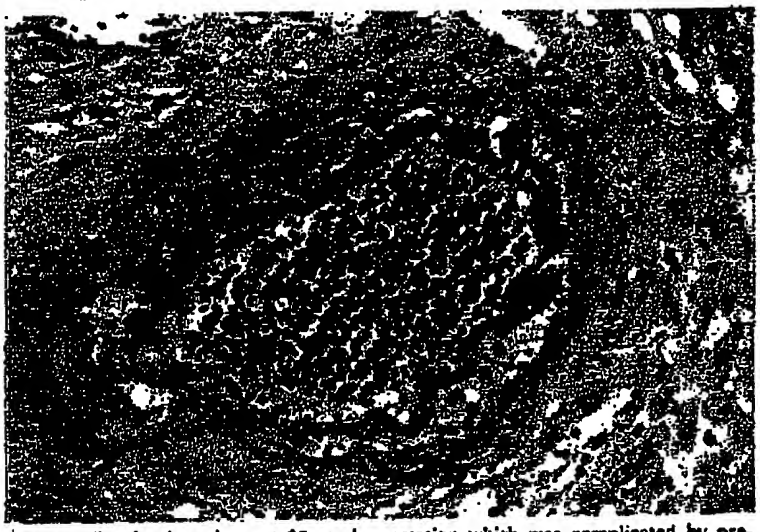

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References

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IN THE MATTER OF
US Patent Application No. 09/380,327
by Robertson et al

EXHIBIT DAC-3

This is Exhibit DAC-3 referred to in the Statutory Declaration dated 2 July 2004 by David Alexander Clark.

David A. Clark

Before me: July 2, 2004 @ Burlington, Ontario

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada

FULVIO DELIBATO
NOTARY PUBLIC
etc.

CELLULAR IMMUNOLOGY 102, 43–51 (1986)

Characterization of the Cellular Basis for the Inhibition of Cytolytic Effector Cells by Murine Placenta

DAVID A. CLARK[1] AND GERARD CHAOUAT

INSERM U 255, Institut Curie, 26 Rue d'Ulm, 75231 Paris Cedex 05, France

Received January 27, 1986; accepted May 2, 1986

Direct suppression of cytolytic effector cell function by cells of the placenta may represent one mechanism that protects the "fetal allograft" from rejection by maternal transplantation immunity. Collagenase disaggregated murine placental cells block target cell lysis by natural killer, lymphokine-activated killer, and (CTL)-type killer cells. This inhibition is reversible and noncompetitive, similar to a previously described inhibitor of CTL found in spleens of mice undergoing an acute graft vs host (GVH) response. Velocity sedimentation separation of placental cells shows that the inhibitory activity is primarily associated with cells that cosediment with nucleated fetal erythrocytes. When these erythrocytes were lysed, an increased number of nonerythrocytic cells could be separated and under this circumstance, inhibitory activity was seen in association with either small white cells or fetal erythrocytes and with large white cells. There may be several cell populations in murine placenta that can inhibit cytolytic effector cells. The possible relevance of direct placental inhibition of cytolytic effectors to protection of the "fetal allograft" is discussed.

INTRODUCTION

The mechanisms explaining nonrejection of the antigen-bearing fetus by the mother during pregnancy appear to lie at the maternofetal interface (1). There is little evidence for systemic immunosuppression sufficient to block immune rejection of infectious agents, tumor allografts, or skin allografts in pregnant females, and allospecific suppressor T cells and "blocking" antibodies that may arise as a consequence of successful pregnancy lack sufficient potency to fully prevent a primary or second-set allograft rejection (2, 3). In contrast, allografts placed at the choriodecidual junction at the intrauterine implantation site enjoy prolonged survival. This local protection occurs even in allosensitized hosts provided there has been a prior pregnancy, an observation that has been interpreted as showing a synergy between local intrauterine suppressor mechanisms and systemic immunological events (4).

It has generally been held that fetal trophoblast cells (chorionic tissue) play a key role in the immunological events at the maternofetal interface. Even though some fetal trophoblast subpopulations may express Class I paternal major histocompatibility complex (MHC) determinants, trophoblast–lymphocyte cross-reactive antigens, trophoblast-specific antigens, oncofetal antigens, and natural killer (NK)-recognition

[1] Associate Professor and MRC Scientist on sabbatical leave from McMaster University, 1200 Main St. West, Hamilton, Ontario, Canada, L8N 3Z5. To whom all correspondence and reprint requests should be addressed.

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structures (5, 6), the trophoblast cells resist lysis by NK cells and by cytotoxic T lymphocytes (CTL) *in vitro* and *in vivo*. When there is apparent fetal rejection with an associated infiltrate of maternal cytotoxic cells, these effectors appear to first pass through or between the trophoblast without causing overt lysis (7). In murine models of spontaneous abortion associated with maternal lymphocytic infiltration, a common feature is a local deficiency of nonspecific suppressor cells; from such cells one can obtain a soluble factor that inhibits the response to interleukin 2 (IL-2) (8–10). Since these suppressor cells appear to be activated or recruited by trophoblast cells (11, 12), one mechanism by which trophoblast may protect the embryo from maternal cytotoxic cell attack is by recruiting and maintaining a suppressor population that prevents the maternal effectors from receiving the IL-2-dependent "help" that they need to proliferate and remain actively cytolytic. NK cells, lymphokine-activated killer (LAK) cells, and specific CTL may all be included among the effector cell population.

A second mechanism that has been postulated by which trophoblast may protect the fetus and itself from being attacked effectively is direct suppression of cytolytic effector cells (13). CTL, NK activity, and antibody-dependent cytotoxic (ADCC) effector cells all appear to be inhibitable by collagenase-digested murine placental cells. The mechanism of this inhibition has not been completely elucidated (13, 14). Although supernatants from placental cells can cause NK effector cell inhibition if the supernatants are first concentrated on an Amicon membrane (15), a cold-target inhibitory mechanism (which could be mediated by soluble ligand) has not been completely excluded. Further, the nature of the inhibitory cell in the crude placental cell digests has not been defined so that it has not been possible to prove that it is the trophoblast cells themselves that are the inhibitors.

In this paper we show that murine placental cells can inhibit the lytic activity of NK, LAK, and CTL effector cells in a reversible and noncompetitive manner. Cell separation studies show that the suppressive activity in crude placental digests cosediments at unit gravity with nucleated erythrocytes (fetal red cells) in the placenta. When these erythrocytes are depleted, inhibitory activity associated with larger sized (and also possibly some smaller sized) white nucleated cells can be demonstrated. Thus, several types of placental cells may be capable of suppressing effector cell activity of which large-sized trophoblast cells may be one subset.

MATERIALS AND METHODS

Mice. C3H/HeJ, Balb/c, Balb/c *nu/nu*, allopregnant (14- to 16-day) C3H mated to DBA/2, and synpregnant C3H mated to C3H were obtained from the IRCS breeding facility at Villejuif.

Preparation of placental cells. Placentae from pregnant mice were carefully peeled from the uterine decidua and the fetus and fetal membranes were removed by dissection. The placentae were then minced with scissors and incubated with Sigma Type V collagenase (1 mg/ml) in RPMI 1640 or α-minimal essential medium (MEM) supplemented also as to contain 10% fetal bovine serum (FBS) (GIBCO), 100 iu/ml penicillin, and 100 μg/ml streptomycin. After 1 hr at 37°C, the placental fragments were pressed gently through a stainless steel screen, washed, and counted in 0.4% trypan blue. In some experiments, the fetal erythrocytes were lysed by resuspension in 0.9% Tris–NH_4Cl at 4°C and were then spun down at 1000 rpm at 4°C, resuspended in

RPMI 1640 without FBS, recentrifuged, and washed once in regular serum-containing medium. Velocity sedimentation separation was carried out at 4°C using a STA-PUT apparatus (John's Scientific, Toronto, Ontario, Canada) for 2 hr in a linear-step gradient of bovine serum albumen (BSA) in phosphate-buffered saline (PBS) as described in detail elsewhere (16).

Effector cells and cytotoxicity assays. NK cell activity was obtained from Balb/c *nu/nu* spleen cells. Single cells suspensions were prepared by homogenizing the spleens in tissue culture medium with 10% FBS in Kontes glass homogenizers. LAKs were prepared by incubating 1×10^6/ml C3H spleen cells in α-MEM + 10% FBS + antibiotics + 5×10^{-5} *M* 2-mercaptoethanol (30 ml in a Falcon 3013 flask set up on end) with phorbol myristate acetate (PMA)-stimulated EL-4 (C15 or F15 lines) supernatant (interleukin 2 (IL-2) source). The IL-2 was kindly provided by Dr. J. P. Kolb. After 6 days at 37°C in 5% CO_2, the cells were harvested, washed once, and resuspended in medium for testing. CTL were generated by incubating 3–4 $\times 10^6$ C3H spleen cells with 1×10^6 irradiated Balb/c spleen cells in α-MEM as described above in Falcon 2057 tubes for 4 to 5 days at 37°C 7% CO_2. The effector cells were tested for their ability to lyse YAC-1 target cells. Briefly, the YAC-1 cells maintained *in vitro* were incubated with $Na_2\,^{51}CrO_4$ 200 μCi for 45 to 60 min at 37°C and were then washed three times. Effector cells were added to 96-well V- or flat-bottom microtrays containing labeled target cells along with placental cells or STA-PUT fractionated placental cells. Depending on the type of effector cells being tested, the assay was terminated after 4 to 20 hr by removing 100 μl of supernatant for counting in an LKB autogamma. Total releasable isotope from the targets was determined by incubation in 4 *N* HCl, and spontaneous release was determined by incubating the YAC targets alone in medium. The percentage specific ^{51}Cr-release value was calculated from the standard formula

$$P = 100\% \times \frac{(\text{cpm with effector cells} - \text{spontaneous})}{(\text{cpm with HCl} - \text{spontaneous})}.$$

The mean and SEM were calculated from three to six replicate wells.

Michaelis–Menten kinetic analysis. The lysis of target cells by cytolytic effector cells may be likened to the action of an enzyme on its substrate (17). In the case of the ^{51}Cr-release assay, the product of the action of the enzyme (killer cell) on the substrate (target cells) is target death and ^{51}Cr release released into the supernatant. It has been possible to analyze the mechanism of inhibition (competitive by cold targets vs noncompetitive) of allospecific CTL by varying the number of target cells added to a fixed number of effector cells and inhibitors and calculating the values for V_{max} and K_m, the Michaelis–Menten constant (17, 18). A similar analysis was performed using placental cells as inhibitors and *nu/nu* or CTL effectors to lyse the YAC targets. The values for V_{max} and K_m were determined by analysis of the relationship of the observed V (number of targets lysed during the assay) to S (substrate concentration—the number of live targets added at the start of the assay). Using a computer program for the nonparametric analytic method of Cornish-Bowden and Eisenthal (18, 19) that provides 95% confidence intervals for the value of each parameter. In *pure* competitive inhibition, V_{max} is unchanged but K_m increases in value whereas in *pure* noncompetitive inhibition, V_{max} is reduced and K_m remains unchanged.

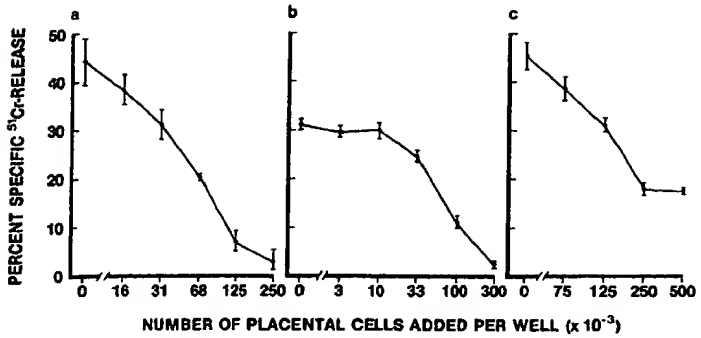

FIG. 1. Effect of C3D2F$_1$ placental cells on lysis of YAK targets. (a) 2.5 × 10^5 Balb/c *nu/nu* spleen cells per well were used as a source of NK activity; (b) C3H spleen cells cultured in IL-2 for 6–7 days were used as a source of LAKs 5 × 10^5/well; (c) C3H spleen cells sensitized to Balb/c cells for 4 days were used as a source of CTL, 5 × 10^5/per well. Data represent mean specific ^{51}Cr release ± 1 SEM. Assays using NK cells were run 18–20 hr and with LAK and CTL, 4–5 hr.

RESULTS

Inhibition of cytolytic effector activity by placental cells. Figure 1 shows that placental cells from C3D2F$_1$ embryos produced a dose-dependent inhibition of NK activity (Fig. 1a), LAK killing (Fig. 1b), and anti-H-2^d CTL (Fig. 1c). In general, the onset of inhibition could be seen when the ratio of placental cells to cytolytic effectors was between 1:10 and 1:5. It has previously been shown that this inhibition is not likely due to nonspecific crowding since thymocytes, liver cells, and embryo cells lacked significant inhibitory effects at the concentrations and inhibitor-to-effector ratios shown in Fig. 1 (13). Thus, there seemed to be some tissue specificity to the potent inhibition produced by placental cells. An antigen-specific inhibition did not appear to be responsible since only CTL have antigen specificity in the classical sense and we found that C3H × C3H placental cells syngeneic to the CTL were also potent inhibitors of CTL lysis (14, data not shown).

Inhibition of cytolysis is reversible and noncompetitive. Previous studies have shown that a non-T cell that inhibits lysis by CTL occurs in the spleens of mice undergoing graft vs host (GVH) reactions and can also be found in the spleen during the CTL reaction following tumor allograft immunization (18). This inhibition was nonantigen specific, reversible on dilution of the mixture of CTL and inhibitor cells, and was noncompetitive. To determine if the placental inhibitor of killer cells behaved in a similar manner, mixtures of NK cells and placental cells or CTL and placental cells were serially diluted to create a titration curve. It can be readily seen that NK lytic activity (Fig. 2a) and CTL activity (Fig. 2b) were inhibited by admixed placental cells at the highest cell concentrations per well, but as the cells were serially diluted, the inhibitory effect decreased so that as much lysis was obtained at lower cytolytic effector-to-target ratios as in the absence of placental cell inhibitors. Therefore, placental inhibition of lysis was reversible, similar to the previously described GVH-associated splenic inhibitor.

To distinguish between competitive inhibition of lysis, as may be produced by unlabeled cells bearing target structures seen by cytolytic cells on the YAC-1 targets, and a noncompetitive inhibition, the number of labeled target cells was progressively

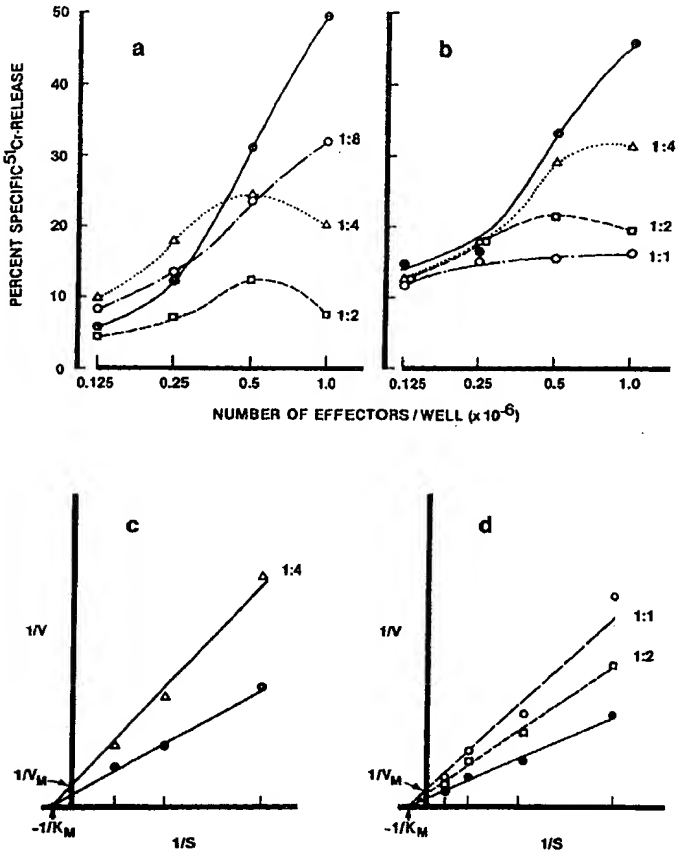

FIG. 2. Placental cells inhibit target cell lysis by a reversible noncompetitive mechanism. (a) *Nu/nu* spleen cells were tested alone (●) or with placental cells at a ratio of 1:2 (□), 1:4 (△), or 1:8 (○), and serially diluted in flat-bottom assay wells. ^{51}Cr-labeled YAC targets were added and the supernatant was harvested after 20 hr. (b) CTL were tested alone (●) or with admixed placental cells in a 1:1 (○), 1:2 (□), or 1:4 (△) ratio. (c) *Nu/nu* spleen cells 10^6 per well (●) or 10^6 per well with 0.25×10^6 placental cells (1:4 ratio) (△) were tested against 2×10^4, 4×10^4, or 8×10^4 target cells and the number of targets lysed in hours (V, velocity of reaction) was determined by multiplying the % ^{51}Cr-release value by the number of targets per well (S, substrate). The data are plotted using the Lineweaver–Burke double reciprocal method, and the values for V_{max} and the Michaelis–Menten constant, K_m, were determined by computer analysis. For *nu/nu* splenocytes alone (●), mean V_{max} (and 95% confidence interval) was 55416 (39942–70104) whereas with placental cells, V_{max} was 36037 (15973–53605), $P < 0.05$. The respective K_m values were 147933 (22601–235800) and 124688 (18288-699776), P = NS. (d) Similar to (c) except that CTL were used and target numbers were 2×10^4, 4×10^4, 8×10^4, and 16×10^4. The V_{max} value for CTL alone (●) was 83226 (53114–89628); CTL + placental cells 1:2 (□), 39638 (30265–57930), $P < 0.05$; and CTL + placental cells 1:1 (○), 27018 (18491–48248). Corresponding K_m values were 154539 (97742–178488), 117466 (82003–214480), P = NS, and 100413 (48734–228928), P = NS. In both (c) and (d), only the V_{max} was significantly reduced by placental cells and there was no increase in K_m.

increased in the assay wells. It was never possible by this method to obtain the same amount of lysis as was produced by the cytolytic cells alone. This is formally shown in the Lineweaver–Burke double reciprocal plots in Figs. 2c and 2d. The maximum

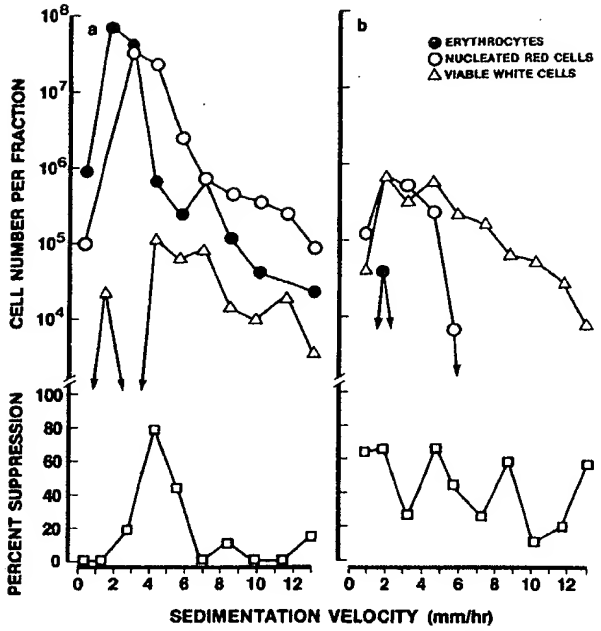

FIG. 3. Velocity sedimentation analysis of placental cells inhibiting target lysis by *nu/nu* splenic NK cells. (a) Separation of C3D3F$_1$ placental cell suspension. 1×10^6 *nu/nu* spleen cells were tested in an overnight ^{51}Cr-release assay alone or with cells from each fraction equivalent to 2.5×10^5 unseparated nucleated red and white cells. Lysis by *nu/nu* alone was 21.9%. (b) Separation of placental cells after removal of nucleated erythrocytes with 0.9% Tris–NH_4Cl. Lysis by *nu/nu* in absence of placental cells was 14%. Placental cells from each fraction equivalent to 2.5×10^5 unseparated cells were tested. (□), % suppression.

lysis here is given by V_{max} as determined by computer analysis of the data. The exact values are provided in the figure legend. It can be seen that placental cells decreased V_{max} without increasing K_m, a pattern of inhibition which is noncompetitive and not due to cold target inhibition (18).

Size of the placental cell responsible for inhibition of lysis by NK cells. Figure 3a shows the velocity sedimentation separation of collagenase-digested placental cells. It can be seen that erythrocytes and nucleated red blood cells (NRBC) were 10–100 times more numerous than viable nucleated white cells. Indeed, in the 3 mm/hr fraction, it was not possible to recognize white cells in the hemocytometer count due to the overwhelming number of cells with red cytoplasm. Interestingly, the peak suppressor activity proved to be associated with small cells with a modal sedimentation velocity of 4 mm/hr rather than with "large" trophoblast cells. To study the properties of the nucleated white cells, it was necessary to deplete the suspension of erythrocytes and this was accomplished using 0.9% Tris–NH_4Cl. Figure 3b shows that a substantial increase in the number of viable nucleated white cells per fraction was achieved, but that a few cells typical of nucleated erythrocytes persisted as did a few adult erythrocytes. There was a change in the distribution of suppressor activity. Suppression was reduced at 4 mm/hr but some activity at 4.5 mm/hr still could be seen. In contrast to Fig. 3a, however, there was now inhibitory activity at 1 mm/hr (which contains

mainly membrane fragments and dead cells) and activity at 9 and 13 mm/hr where only nucleated white cells could be seen. We concluded that nonerythroid cells and cell membranes were capable of inhibiting NK lytic activity.

DISCUSSION

The data in this paper confirm that murine placental cells can inhibit target lysis by a variety of cytolytic effector cells. The inhibition is noncompetitive and reversible. The nature of the placental cell responsible for inhibition of lysis was investigated using velocity sedimentation. It had been predicted that the inhibitor would be a large trophoblast cell, but the data using crude placental cell preparations showed that there were few nucleated white cells in the suspension and that the suppressive activity cosedimented with nucleated erythrocytes (NRBC or fetal red cells). When the NRBC were lysed, suppressive activity could be detected in other cell fractions, notably at 9 and 13 mm/hr. Placental cells also produce a soluble factor that inhibits the generation of CTL *in vitro* and a GVH *in vivo* and in a preliminary experiment, we have found this factor to derive from the 9 mm/hr cells (G. Chaouat and D. Clark, unpublished). Thus, we suspect that the large cells in placental digests may be trophoblast. It is, however, not formally possible to prove such an assertion since there are, as yet, no reagents that specifically distinguish murine trophoblast cells from nontrophoblast. Rossant and Croy (20) have shown that approximately 15% of the "spongiotrophoblast" cells in the mouse placenta are *maternally* derived, and Zuckerman has shown that Percoll-purified murine trophoblast cells are large in size and produce cold target inhibition that is antigen specific (21). Thus, the inhibition shown using placental cells syngeneic and allogeneic to the effector cells may not be due primarily to trophoblast cells (14). One potential caveat arises from the fact that Zuckerman dispersed his placental cells with dispase rather than collagenase and we have found that certain types of enzymes including dispase can destroy nonspecific suppressor activity (13, 14, manuscript in preparation). Further, large-sized non-T cells with nonspecific suppressive activity against CTL can occur in the spleens of alloimmunized adult mice (18). It is possible that similar cells may infiltrate the placenta during pregnancy. Since such cells should express maternal but not paternal MHC determinants, it may be possible to detect inhibitors of maternal origin. These experiments have not yet been completed. The 4 mm/hr inhibitory cells are also as yet unidentified. When NRBC were lysed, the magnitude of suppression at 4 mm/hr compared to cells of other sizes decreased (Fig. 3b). Nevertheless, some fetal red cells persisted in this part of the STA-PUT profile. These findings suggest that fetal erythrocytes may be responsible for the majority of the suppression in unseparated placental cell suspensions. However, in Fig. 3b a decrease in suppression occurred at 3 mm/hr compared to 4 mm/hr unassociated with any change in the number of fetal red cells whereas viable nucleated cell numbers decreased. Kolb *et al.* (14) isolated fetal red cells using Ficoll gradients and could document no significant suppressive activity. Taken together, these data suggest that if fetal red cells act as inhibitors of lysis, a subpopulation must be responsible. One cannot exclude the possibility that a very potent nucleated white cell at 4 mm/hr may be primarily responsible. Cell sorting using a fluorescence-activated cell sorter (FACS) will likely be needed to conclusively establish the nature of the suppressor cell population that sediments at 4 mm/hr. Further studies will also be needed to determine if a nucleated white cell suppressor at 4 mm/hr is of fetal or maternal origin.

The velocity sedimentation profile suggests that membrane fragments can have some suppressive activity. The origin of these fragments is unknown, but NRBC have likely contributed. It is interesting that membrane fragments prepared from the spleens of mice undergoing a GVH reaction retained suppressive activity for CTL (18). A soluble factor can be obtained that blocks effector cell activity, and the placental inhibitor appears to contain carbohydrate (22). Interestingly, Pimlott and Miller have recently shown that certain types of glycopeptide extracts of membranes can block the recognition of targets by CTL (23), but these extracts showed MHC specificity unlike the inhibitory effect of placental suppressors.

There is some information to suggest that suppression of cytolytic effector activity by placental cells may be biologically significant. It had been previously shown that CBA × DBA/2 fetuses with a tendency to spontaneously abort could be "rescued" by vaccination of the mother with Balb/c cells and that this elimination of abortion proneness was associated with a greater ability of the placental cells to inhibit cytolytic effector activity. *Mus caroli* embryos placed in the uterus of *M. musculus* recipients are almost invariably aborted before Day 15.5 of pregnancy whereas *M. musculus* embryos were not so rapidly destroyed when gestated in the *M. caroli* uterus (25). The nonreciprocal nature of this system may in part by due to the occurrence of preexisting sensitization to *M. caroli* antigens in *M. musculus* recipients (26). In addition, trophoblast does not seem to be able to recruit decidua-associated suppressor cells across the species barrier (11, 12). However, a third factor that may be important is a relative deficiency in the ability of the *M. caroli* placental cells to inhibit CTL and LAK (26) and NK cell effector activity (unpublished data). This deficiency is similar in magnitude to that seen in abortion-prone CBA × DBA/2 F_1 placentae (24). Definitive identification of the origin and type of suppressor in the placental cell suspension will be needed to fully evaluate the possible significance of these findings in intra- and interspecies pregnancy failure.

Great emphasis has been placed on the resistance of trophoblast cells to cytolysis by effector cells in explaining the mechanism of fetal protection at the maternofetal interface (13). However, as already noted, in models of spontaneous abortion, maternal lymphoid cells appear to traverse the trophoblast without lysing it (7). These observations imply that lysis of trophoblast cells may not be essential for abortion. An interesting parallel is the ability of sensitized lymphocytes to traverse the blood–brain barrier whereas immunocompetent but unsensitized lymphocytes fail to do so (27). A further piece of evidence that resistance of putative trophoblast cells to lysis is independent of their putative ability to inhibit lysis by NK cells comes from the finding that trypsinization that eliminates the ability of placental cell trophoblast to block lysis by NK cells does not render them susceptible to lysis by murine, rat, or human NK activity (14, unpublished data). Further, it is of interest that several murine teratocarcinoma cell lines that produce CTL and NK inhibitory factor are nonetheless rejected *in vivo* (28). Whether trophoblast cells become susceptible to CTL or LAK lysis is unclear in part because there is no method yet available to determine if it is actually trophoblast cells that are being lysed in trypsin-treated placental cell suspensions (7). However, both the recruitment of decidua-associated suppressor activity and the direct inhibition of effector cells, perhaps by an action on their surface properties or motility, represent potential functional effects of trophoblast cells (that would inhibit lytic activity). If these functions of trophoblast were disturbed, without having to lyse the trophoblast cells, there would nonetheless be a breakdown of defences against maternal effector mechanisms. Whether such a dysfunction in trophoblast is

biologically or immunologically inducible (as it relates to models of murine abortion) (29, 30) remains unknown. In understanding the mechanisms of protection of the fetus, it will likely be crucial to be able to isolate and accurately identify murine trophoblast cells and then to determine the mechanisms which interfere with their normal physiologic defence functions against maternal immunity.

REFERENCES

1. Clark, D. A., Slapsys, R., Croy, B. A., Krcek, J., and Rossant, J., *Amer. J. Reprod. Immunol.* **5**, 78, 1984.
2. Chaouat, G., and Monnot, P., *Amer. J. Reprod. Immunol.* **6**, 5, 1984.
3. Nagarkatti, P. S., and Clark, D. A., *J. Immunol.* **131**, 638, 1983.
4. Sio, J. O., Quebbeman, J. F., and Beer, A. E., *J. Reprod. Immunol. (Suppl).* **1**, 36, 1983.
5. Faulk, W. P., and McIntyre, J. A., *Immunol. Rev.* **75**, 139, 1983.
6. Lala, P. K., Chatterjee-Hasrouni, S., Kearns, M., Montgomery, B., and Colavincenzo, V., *Immunol. Rev.* **75**, 87, 1983.
7. Clark, D. A., Slapsys, R. M., Croy, B. A., and Rossant, J., *Immunol. Today* **5**, 111, 1984.
8. Clark, D. A., Chaput, A., and Tutton, D., *J. Immunol.* **136**, 1668, 1986.
9. Clark, D. A., Slapsys, R. M., Croy, B. A., and Rossant, J., *J. Immunol.* **131**, 540, 1983.
10. Clark, D. A., Chaput, A., Walker, K., and Rosenthal, K. L., *J. Immunol.* **134**, 1659, 1985.
11. Clark, D. A., and Slapsys, R. M., *In* "Advances in Obstetrics and Gynecology" (A. E. Beer and V. Toder, Eds.), Vol. 14, pp. 44–53. Karger, Basel, 1985.
12. Slapsys, R. M., Beeson, J., and Clark, D. A., *Fed. Proc.* **44**, 1880, 1985.
13. Kolb, J. P., Chaouat, G., and Chassoux, D., *J. Immunol.* **132**, 2305, 1984.
14. Chaouat, G., and Kolb, J. P., *J. Immunol.* **135**, 215, 1985.
15. Kolb, J. P., and Chaouat, G., *Immunobiology* **163**, 113, 1982.
16. Miller, R. G., *In* "New Techniques in Biophysics and Cell Biology" (R. H. Pain and B. J. Smith, Eds.), Vol. 1, pp. 87–112. Wiley, London, 1973.
17. Harley, E., M.Sc. Thesis, University of Toronto, 1976.
18. Clark, D. A., Phillips, R. A., and Miller, R. G., *Cell. Immunol.* **34**, 25, 1977.
19. Cornish-Bowden, A., and Eisenthal, R., *Biochem. J.* **139**, 721, 1974.
20. Rossant, J., and Croy, B. A., *J. Embryol. Exp. Morphol.* **86**, 177, 1985.
21. Zuckerman, F., Ph.D. Thesis, University of Texas, Health Science Center at Dallas, 1986.
22. Chaouat, G., Kolb, J. P., Wegmann, T. G., Chaffaux, S., and Riviere, M., *In* "Immunoregulation and Fetal Survival" (T. G. Wegmann and T. Gill, Eds.). Oxford Univ. Press, New York, in press.
23. Pimlott, N. J. G., and Miller, R. G., *J. Immunol.* **133**, 1763, 1984.
24. Chaouat, G., Kolb, J. P., and Riviere, M., *Ann. Immunol. (Inst. Pasteur)* **135D**, 301, 1984.
25. Croy, B. A., Rossant, J., and Clark, D. A., *J. Reprod. Fertil.* **74**, 479, 1985.
26. Clark, D. A., Croy, B. A., Rossant, J., and Clark, D. A., *J. Reprod. Fertil.*, in press.
27. Barker, C. F., and Billingham, R. E., *Adv. Immunol.* **25**, 11, 1977.
28. Chaouat, G., Kolb, J. P., Riviere, M., and Lankar, D. *J. Reprod. Immunol.* (Suppl.) **1**, 177, 1986.
29. Clark, D. A., *Immunol. Lett.* **9**, 239, 1985.
30. Clark, D. A., Chaput, A., Slapsys, R. M., Brierley, J., Daya, S., and Allardyce, R., *In* "Immunoregulation and Fetal Survival" (T. G. Wegmann and T. Gill, Eds.). Oxford Univ. Press, New York, in press.

Identification of gonadotropin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. *Biol. Reprod.* 37, 1075–1082.

Fowler, P.A., Messinis, I.E. & Templeton, A.A. (1989) Gonadotrophin surge-attenuating factor (GnSAF) activity in human follicular fluid is different from inhibin. *J. Reprod. Fert., Abstr. Ser.* No. 4, 30, abstr.

Fraser, H.M. & Lunn, S.F. (1989) Rapid inhibitory effects of an LHRH agonist implant on the oestrogen-induced LH surge and the induction of a defective luteal phase after an agonist-induced ovulation in the macaque. *Hum. Reprod.* 4, 506–512.

Fraser, H.M., Abbott, M., Laird, N.C., McNeilly, A.S., Nestor, J.J., Jr & Vickery, B.H. (1986) Effects of an LH-releasing hormone antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (*Macaca arctoides*). *J. Endocr.* 111, 83–90.

Fraser, H.M., Sandow, J., Seidel, H. & von Rechenberg, W. (1987) An implant of a gonadotropin releasing hormone agonist (buserelin) which suppresses ovarian function in the macaque for 3–5 months. *Acta endocr., Copenh.* 115, 521–527.

Fraser, H.M., Robertson, D.M. & de Kretser, D.M. (1989) Immunoreactive inhibin concentrations in serum throughout the menstrual cycle of the macaque: suppression of inhibin during the luteal phase by treatment with an LHRH antagonist. *J. Endocr.* 121, R9–R12.

Glasier, A., Thatcher, S.S., Wickings, E.J., Hillier, S.G. & Baird, D.T. (1988) Superovulation with exogenous gonadotropins does not inhibit the luteinizing hormone surge. *Fert. Steril.* 49, 81–85.

Land, R.B., Morris, B.A., Baxter, G., Fordyce, M. & Forster, J. (1982) Improvement of sheep fecundity by treatment with antisera to gonadal steroids. *J. Reprod. Fert.* 66, 625–634.

Littman, B.A. & Hodgen, G.D. (1984) Human menopausal gonadotropin stimulation in monkeys: blockade of the luteinizing hormone surge by a highly transient ovarian factor. *Fert. Steril.* 41, 440–447.

McLachlan, R.I., Robertson, D.M., Healy, D.L., Burger, H.G. & de Kretser, D.M. (1987) Circulating immunoreactive inhibin levels during the normal human menstrual cycle. *J. clin. Endocr. Metab.* 65, 954–961.

McLachlan, R.I., Robertson, D.M., Healy, D.L., de Kretser, D.M. & Burger, H.G. (1986) Plasma inhibin levels during gonadotrophin-induced ovarian hyperstimulation for IVF: a new index of follicular function? *Lancet*, 1233–1234.

Messinis, I.E. & Templeton, A.A. (1987) Endocrine and follicle characteristics of cycles with and without endogenous luteinizing hormone surges during superovulation induction with pulsatile follicle-stimulating hormone. *Hum. Reprod.* 2, 11–16.

Messinis, I.E. & Templeton, A.A. (1989) Pituitary response to exogenous LHRH in superovulated women. *J. Reprod. Fert.* 87, 633–639.

Robertson, D.M., Giacometti, M., Foulds, M., Lahnstein, J., Goss, N.H., Hearn, M.T.W. & de Kretser, D.M. (1989) Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. *Endocrinology* 125, 2141–2149.

Robertson, D.M., Tsonis, C.G., McLachlan, R.I., Handelsman, D.L., Leask, R., Baird, D.T., McNeilly, A.S., Hayward, S., Healy, D.L., Findlay, J.K., Burger, H.G. & de Kretser, D.M. (1988a) Comparison of inhibin immunological and in vitro biological activities in human serum. *J. clin. Endocr. Metab.* 119, 438–443.

Robertson, D.M., Hayward, S., Irby, D., Jacobsen, J., Clarke, L., McLachlan, R.I. & de Kretser, D.M. (1988b) Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. *Molec. cell. Endocrinol.* 58, 1–8.

Schenken, R.S. & Hodgen, G.D. (1983) Follicle stimulating hormone induced ovarian hyperstimulation in monkeys: blockade of the luteinizing hormone surge. *J. clin. Endocr. Metab.* 57, 50–55.

Stillman, R.J., Williams, R.F., Lynch, A. & Hodgen, G.D. (1983) Selective inhibition of follicle stimulating hormone by porcine follicular fluid extracts in the monkey: effects on midcycle surges and pulsatile secretion. *Fert. Steril.* 40, 823–828.

Tsonis, C.G., Messinis, I.E., Templeton, A.A., McNeilly, A.S. & Baird, D.T. (1988) Gonadotropic stimulation of inhibin secretion by the human ovary during the follicular and early luteal phase of the cycle. *J. clin. Endocr. Metab.* 66, 915–921.

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Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy

G. Chaouat*, E. Menu*, D. A. Clark†, M. Dy‡, M. Minkowski‡ and T. G. Wegmann§

**U 262 INSERM, Clinique Universitaire Baudelocque, 123 Bd de Port Royal, 75674 Paris Cedex 14, France; †McMaster University, Department of Medicine, Hamilton, Ontario, Canada L8N 3Z1; ‡U 25 INSERM, Hopital Necker, 149 rue de Sèvres, 75015 Paris, France; and §Department of Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7*

Summary. In this study, we examined the effect of injecting various cytokines. We report here that tumour necrosis factor (TNF)α, γ-interferon and interleukin 2 (IL-2) can, in some circumstances, increase fetal resorption rates in abortion-prone (CBA/J × DBA/2) and non-abortion prone (CBA/J × BALB/c,C3H × DBA/2) matings: 1000 units TNF enhanced resorptions from 43 to 79% in CBA × DBA/2, from 7 to 89% in CBA × BALB/c, from 5 to 47% in C3H × DBA/2. The effect was both gestational age- and dose-dependent. Gamma interferon and R-IL-2 enhanced resorptions from 38 to 68% and 76% respectively in the CBA/J × DBA/2 mating combination, whereas the rates in CBA/J × BALB/c matings were enhanced from 6 to 44% and 55%. Lipopolysaccharride (LPS), which is known to lead to the release of TNF-α, had a similar effect, leading to gestational age- and dose-dependent enhancement of resorptions up to 100%.

However, cytokines of the CSF family, including IL-3 and GM-CSF, increased the chances of fetal survival when injected into abortion-prone mice, e.g. reducing resorption rates in the abortion-prone CBA/J × DBA/2 mating combination from 55 to 22% (IL-3), and 47 to 8% (GM-CSF). They also increased fetal and placental weight and, in particular, expanded the spongiotrophoblast zone in the placenta. The latter observations may be due to a direct trophic influence on placental cells, perhaps through a cytokine cascade, or an indirect effect due to inhibition of natural killer (NK)-like cells, or both. Whatever the mechanism, these results may find practical application in influencing reproductive outcome in women and other species.

Keywords: abortion; mice; lymphokines; induction; treatment

Introduction

There is increasing evidence that some recurrent abortions in women might be preventable by immunization of the mother against paternal or third party alloantigens (Taylor & Faulk, 1981; Mowbray *et al.*, 1985). Ethical concerns limit the extent of studies on this subject in women but there now exist several models of pregnancy failure in mice and its correction by lymphocyte alloimmunization.

The best studied model is the mating of CBA females with DBA/2 males: CBA/J, but not C3H-HeJ, females have an abnormal resorption rate when mated with DBA/2 males (Clark *et al.*, 1980), but when CBA/J females are mated with BALB/c males, the fetal resorption rate is normal (Chaouat *et al.*, 1983). In this system, there is suggestive evidence that abortion is mediated by asialo GM1-positive natural effector cells (Chaouat *et al.*, 1987; Baines & de Fougerolles, 1988). If

CBA/J females are immunized against BALB/c lymphocytes before mating, the high resorption rates in CBA/J mice mated with DBA/2 males are prevented (Chaouat *et al.*, 1983, 1987, 1988a, b, c) and there is no longer accumulation of asialo Gm1+ cells in the uterus (Gendron & Baines, 1988). Protection against abortion can be adoptively transferred by CBA/J anti-BALB/c immune T cells, or T cells from the spleens of CBA/J mice that were multiparous by repeated mating to BALB/c males (Chaouat *et al.*, 1985). Furthermore, T cell-derived cytokines such as IL-3, GM-CSF and CSF-1 were strongly supportive of the in-vitro proliferation of placental cells (Athanassakis *et al.*, 1987; Armstrong & Chaouat, 1989), and, conversely, tumour necrosis factor (TNF) -α and -β proved to be cytostatic for these placental cells *in vitro*. In addition, in-vivo depletion of T cells enhanced resorption rates in CBA females mated with DBA/2 males as well as in the non-abortion prone CBA × BALB/c mating (Chaouat *et al.*, 1988a). Further support for a trophic role of activated T cells *in vivo* came from the observation that depletion of T cells in the autoimmune MRL lpr/lpr strain corrected placental hypertrophy and placental phagocytic activity was then normal. This observation was corroborated by the finding that transfer of cells from MRL lpr/lpr mice into H-2k-compatible CBA/J females prevented the CBA × DBA/2 pregnancy from showing increased fetal resorption (Chaouat *et al.*, 1988a, b). Since these mice are known to produce excess IL-3, and autoantibodies with IL-3-like activity, and based on the aforementioned evidence, we decided to test directly various lymphokines, all of them products from activated T cells, for their effect on fetal resorption in CBA/J mice mated with DBA/2 males.

Materials and Methods

Mice. CBA/J mice were obtained from Iffa Credo (L'Arbresle, France) at the age of 8 weeks. They were kept for another 2 weeks in our animal facility before mating. The day of observing a vaginal plug was termed Day 0·5 of pregnancy.

Since the rate of resorption increases with ageing or environmental conditions (Chaouat *et al.*, 1987, 1988a; Hamilton & Hamilton, 1987) mice were in some cases aged a further 5 weeks before mating in a conventional room where abortions were known to be higher (Chaouat *et al.*, 1987, 1988a) than in the gnotobiotic facility. C3H/HeJ, C57BL/6, DBA/2 and BALB/c.J mice were obtained from the IRSC, Villejuif, France, at the age of 8 weeks, and also maintained for 2 weeks before use.

For experiments performed in Canada 6–8-week-old CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained until 12 weeks of age when they were mated.

Fetal resorptions were assessed on Day 14·5 of pregnancy, unless otherwise stated. In some experiments, animals were allowed to deliver to confirm the anti-abortive effect by direct assessment of viable offspring. This technique suffers from the fact that some pregnant mothers eat their offspring and placentae immediately after birth: the mean litter size was therefore determined on Day 1, as the 'surviving' litter size. Animals which had clearly eaten their progeny were excluded from the experiment. There was no indication that any treatment which we employed had an influence on this type of behaviour.

Lymphokines and reagents. Purified GM CSF was obtained from the P338 D1 cell line as described by Dy *et al.* (1987). Recombinant murine GM-CSF was purchased from Genzyme Inc. (Boston, MA, USA) directly or via Tebu France (Le Perray, Yvelines, France). Recombinant human GM-CSF (produced in CHO cells) was a kind gift from the Genetics Institute, Boston, MA, USA. All recombinant materials were purified to homogeneity by the manufacturers, and did not contain other lymphokines or lipopolysaccharide (LPS) that might interfere with the assay. Lipopolysaccharide (Difco, via Sigma France, itself via OSI France, Paris, France) extracted from *Salmonella enteritidis* was from a batch pretested in various LPS and LPS/TNF assays, and was a kind gift of Dr M. Parand, Institut Biomédical des Cordeliers, Paris, France. Recombinant human TNF-α, murine and human IL-2, and murine γ-interferon were kindly donated by Dr J. Wietzerbin, Institut Curie, Paris, France. Rabbit polyclonal antibody against murine GM-CSF was kindly donated by the Glaxo Institute for Molecular Biology, Geneva, Switzerland. Purified IL-3 was prepared from the WEHI cell line as described by Dy *et al.* (1987).

LPS was injected i.v. at 1 μg on Day 7 or 12, or 3 μg on Day 15. Mice were killed on Day 12 when injection was performed on Day 7, on Day 16 for injection performed on Day 12, or Day 19 for injection performed on Day 15. For TNF experiments, each group was injected i.v. with 1000 units of human recombinant TNF-α or saline on Day 7·5 of gestation, and mice were examined on Day 14·5 for fetal resorption. Alternatively, 1000, 2000 or 3000 units TNF were injected on various days of gestation and mice were checked on Day 12 (injections until Day 10·5) or 17 (injection on Day 13·5) or 19·5 (just before parturition) for injection on Day 17·5. Gamma interferon was given at 1500 units i.p. on Day 7·5 of gestation, and R-IL-2 was given at 2000 units i.p. on Days 6·5, 8·5 and 10·5 of gestation. Mice were killed on Day 14·5. Other mice received 600 units IL-3 i.p. on Day 6·5, 8·5 or 10·5 and were killed on Day 14·5.

GM-CSF was injected i.p. as (a) 400 HCSF units in 0·2 ml saline on Days 6·5, 8·5 or 10·5 or (b) 200 HCSF units in phosphate-buffered saline (PBS) on Day 6·5, 8·5 or 10·5.

For dose–response experiments 200, 400, 1000 or 2500 units of GM-CSF were injected i.p. in PBS. In some cases, GM-CSF was dissolved in medium RPMI 1640 before injection. For the experiments that were conducted in Canada with recombinant human GM-CSF (Genetic Institute) and murine GM-CSF (Genzyme), a single dose of 200 units was injected on Day 7·5 of pregnancy.

Spongiotrophoblast width. To estimate spongiotrophoblast width (Table 7), spongiotrophoblasts were sliced from fresh placentae with a surgical lance, then snap-frozen and measured by calipers in mm. The labyrinthine zone was also measured but the enlargement was not statistically significant in the limits of these experiments (number of placentae studied) and the data are therefore not shown.

Statistics. Student's *t* tests (placental width, fetal and placental weights) and χ^2 analysis (% resorptions) were performed to test the significance of treatment effects.

Results

Negative effects on pregnancy outcome

LPS from *Salmonella enteritidis* has been shown to induce complete fetal death in several strains of mice at a dose of 1 μg per mouse when given i.v. on Day 7·5 or Day 12·5 (a dose of 3 μg per mouse is required for abortion on Day 15–18) (Parand & Chedid, 1964), irrespective of the strain.

Table 1 shows typical experiments that confirm these data for various mating combinations of mice which are not abortion prone, as well as in the CBA × DBA/2 abortion-prone mice.

Direct measurement of TNF-α in the decidua confirmed that abortion was correlated with an enhancement of TNF levels (data not shown, and Chaouat *et al.*, 1990), in agreement with previous data (Parand & Chedid, 1964; Parand, 1987; Gendron *et al.*, 1989).

Table 1. Induction of fetal resorption (%, with no. viable, no. resorbing in parentheses) by lipopolysaccharide in mice of various mating combinations injected on Day 7, 12 or 15

Mating combination	Control mice	Treated mice*		
		1 μg i.v.		3 μg i.v.
		Day 7	Day 12	Day 15
C3H × BALB/c	6 (43, 2)	98 (1, 45)	100 (0, 48)	91 (4, 41)
C57BL/6 × DBA/2	6 (44, 3)	96 (1, 42)	98 (1, 39)	95 (2, 40)
C57BL/6 × BALB/c	8 (47, 4)	100 (0, 48)	96 (2, 53)	98 (1, 50)
CBA/J × BALB/c	10 (45, 4)	100 (0, 51)	100 (0, 48)	92 (4, 48)
CBA/J × DBA/2	38 (17, 28)	100 (0, 45)	100 (0, 49)	100 (0, 43)
C3H × DBA/2	5 (39, 2)	100 (0, 39)	100 (0, 40)	100 (0, 45)

*Mice were killed on Day 12 (injection on Day 7), Day 16 (injection on Day 12) or Day 19 (injection on Day 15).

All treatment groups showed significantly more resorptions than in the respective control groups, $P < 0{\cdot}001$ (χ^2 tests).

When 1000 units of recombinant human TNF-α were injected on Day 7·5 i.p., a significant increase in fetal resorption rates was observed in CBA × DBA/2 mice as well as in the non-abortion-prone CBA × BALB/c and C3H × DBA/2 combinations. The effect was still significant when TNF-α was given on Days 9 and 10. However, to obtain a comparable level of fetal resorption rate, a higher dose of TNF was required on Days 13 and 17, although 1000 units significantly enhanced the fetal resorption rates above background levels (Table 2).

Poly(I).Poly(C12U), an inducer of γ-interferon production and an activator of natural killer cells (NKs) and lymphokine-activated killer cells (LAKCs), is known to increase fetal resorption rates (Baines & de Fougerolles, 1988; Chaouat *et al.*, 1988a, 1989a, b, c; R. Kinsky, G. Lepage,

Table 2. Induction of fetal resorptions (no. viable, no. resorbing) in mice by human recombinant TNF-α

Exp.	Mating combination	Control mice, % resorptions	Treated					
			Days of injection	% Resorption	Days of injection	% Resorption	Days of injection	% Resorption
1 (1000 Units)†	CBA × DBA/2	43 (12, 19)	7·5	79 (6, 22)*	—	—	—	—
	CBA × BALB/c	7 (26, 2)	7·5	89 (5, 39)*	—	—	—	—
	C3H × DBA/2	5 (34, 2)	7·5	47 (15, 17)*	—	—	—	—
2 (1000 Units)‡	CBA × DBA/2	36 (39, 22)	0·5	31 (43, 19)	3·5	83 (6, 30)*	—	—
			5·5	67 (22, 45)*	7·5	83 (11, 55)*	—	—
			9·5	88 (8, 58)*	10·5	75 (14, 43)*	—	—
			13·5	52 (35, 38)	17·5	47 (30, 27)	—	—
3	CBA × DBA/2	39 (33, 21)						
1000 Units†			7·5	89 (5, 41)*	13·5	60 (18, 26)*	17·5	51 (28, 29)
2000 Units†			7·5	100 (0, 42)*	13·5	94 (3, 45)*	17·5	57 (22, 29)
3000 Units†			7·5	100 (0, 48)*	13·5	59 (25, 36)*	17·5	85 (8, 45)*

†Killed Day 14·5.
‡Killed Day 12 (injections on Days 0·5–10·5), Day 17 (injections on Day 13·5) or Day 19·5 (injections on Day 17·5).
*$P < 0·05$ compared with control value for that Exp.

M. N. Thang & G. Chaouat, unpublished). We therefore also tested the effects of treatment with γ-interferon, and recombinant murine IL-2, a LAKCs and NK cell activator. Both treatments enhanced fetal resorption in the CBA × DBA/2 and CBA × BALB/c mating combinations (Table 3).

Table 3. Enhancement of fetal resorptions (%, no. viable, no. resorbing) by murine recombinant γ-interferon (1500 Units on Day 7·5) and murine recombinant IL-2 (2000 Units on Days 6·5, 8·5 and 10·5)

Mating combination	Control mice	Treated mice†	
		γ-Interferon	IL-2
CBA/J × DBA/2	38% (18, 11)	68% (8, 17)*	76% (8, 17)*
CBA/J × BALB/c	6% (31, 2)	44% (18, 14)*	55% (15/18)*

†Killed on Day 14.
*$P < 0·05$ compared with control.

Positive effects on pregnancy outcome

When IL-3 was injected i.p. fetal resorption rates were decreased in CBA × DBA/2 pregnancies whereas in the same experiment treatment with LPS or recombinant human TNF had the opposite effect (Table 4).

Table 4. Reduction of CBA × DBA/2 fetal resorption by murine IL-3 from WEHI cells

Exp.	Control mice			Treated mice†			
	% Resorbing	Placental wt (mg)	Fetal wt (mg)	Treatment	% Resorbing	Placental wt (mg)	Fetal wt (mg)
1	52 (14, 13)	102	121	IL-3‡	28 (15, 6)*	125*	141*
2	55 (17, 21)	—	—	IL-3†	22 (25, 7)*	—	—
3	46 (22, 19)	—	—	IL-3†	19 (30, 7)*	—	—
				LPS§	88 (3, 22)*	—	—
				H-rTNF-α¶	93 (2, 27)*	—	—
				PBS	54 (18, 21)	—	—

†Killed on Day 14·5.
‡600 Units on Days 6·5, 8·5 and 10·5.
§0·1 μg in PBS on Days 6·5, 8·5 and 10·5.
¶500 Units on Days 6·5, 8·5 and 10·5.
*$P < 0·05$ compared with controls.

Treatment with 200 or 400 units of purified natural GM-CSF decreased CBA × DBA/2 fetal resorption rates (Table 5). When different doses of GM-CSF were tested, dosages of >1000 Units per injection were ineffective (Table 5). In fact, 3 × 2500 HCSF units sometimes enhanced fetal resorptions, although most often the enhancement was not significant, and at best P values were $<0·02$ (data not shown).

Treatment with recombinant murine GM-CSF (Genzyme) given on the same schedule at low doses was also protective (Table 6).

Both forms of murine GM-CSF not only affected fetal survival, but also enhanced placental (and fetal) weights (Tables 5 and 6). The enhancement appeared to depend markedly upon additional spongiotrophoblast width as a result of treatment (see Table 6). No significant size enhancement was traced in the labyrinth (data not shown).

Table 5. Reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by purified murine GM-CSF from P338 D1

Exp.	Mating combination	Control mice: % Resorbing	Control mice: Placental wt (mg)	Control mice: Fetal wt (mg)	Treated mice† Treatment‡	Treated mice†: % Resorbing	Treated mice†: Placental wt (mg)	Treated mice†: Fetal wt (mg)
1	CBA × DBA/2	47 (27, 24)	95	118	GM-CSF400	8 (44, 4)*	136*	209*
	CBA × BALB/c	8 (37, 3)	138	211				
2	CBA × DBA/2	37 (17, 10)	—	—	PBS	51 (15, 6)	—	—
					GM-CSF200	7 (29, 2)*	—	—
3	CBA × DBA/2	38 (26, 16)	—	—	GM-CSF200	17 (38, 8)*	—	—
					GM-CSF400	24 (32, 10)*	—	—
					GM-CSF1000	32 (27, 13)	—	—
					GM-CSF2500	53 (18, 20)	—	—

†Killed on Day 14·5.
‡GM-CSF injected on Days 6·5, 8·5 and 10·5 at dose indicated.
*$P < 0.05$ compared with controls.

Table 6. Reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by recombinant murine and human GM-CSF

Exp.	Mating combination	Control mice: % Resorbing	Control mice: Plac. wt (mg)	Control mice: Fetal wt (mg)	Control mice: Spongiotrophoblast width (mm × 10^{-1})	Treated mice† Treatment‡	Treated mice†: % Resorbing	Treated mice†: Plac. wt (mg)	Treated mice†: Fetal wt (mg)	Treated mice†: Spongiotrophoblast width (mm × 10^{-1})
1	CBA × DBA/2	52 (14, 13)	102	122	24·8	GM-CSF from P338 D1	24 (41, 13)*	140*	205*	33·5*
						R murine GM-CSF	30 (33, 14)*	133*	200*	33·1*
	CBA × BALB/c					R murine GM-CSF	17 (24, 5)*	141*	193*	30·6*
2	CBA × DBA/2	66 (11, 21)	—	—	—	R murine GM-CSF	10 (18, 2)*	—	—	—
	CBA × BALB/c	18 (29, 6)	—	—	—					
3	CBA × DBA/2	29 (43, 150)	—	—	—	Human R GM-CSF§	18 (32, 150)*	—	—	—
						R murine GM-CSF§	21 (51, 247)*	—	—	—

In Exps 1 and 2 mice were aged (see 'Methods'). Experiment 3 was carried out in Canada.
†Killed on Day 14·5.
‡On Days 6·5, 8·5 and 10·5.
§On Day 7·5.
*$P < 0.05$ compared with controls.

Similar experiments conducted in Canada used a recombinant human GM-CSF (Genetic Institute) and the same murine GM-CSF (Genzyme), but the control levels of resorption were much lower. A single dose of 200 units of either form of GM-CSF on Day 7·5 of pregnancy was minimally effective (Table 6). The murine GM-CSF stimulated placental cell growth *in vitro* but the human recombinant GM-CSF was inactive in this regard (data not shown, and Clark *et al.*, 1990). High doses of the human GM-CSF (10 000–30 000 units/mouse) were ineffective, similar to the data shown in Table 5.

Since P 338 D1 purified material contained traces of IL-1 and IL-3, it was important to ascertain that the effects of P 338 D1 supernatant and recombinant GM-CSF were mediated by GM-CSF itself and not by some contaminant in the preparation. Recombinant murine GM-CSF, or P 338 D1 material, was passed through a column containing normal rabbit serum coupled to CNBr-activated Sepharose 4B (Pharmacia, Pharmacia-LKB, Les Ulis, France) or similarly prepared rabbit anti-murine GM-CSF. The specific antiserum retained the fetal protective effect, while the control column eluant did not (Table 7).

Table 7. Removal of reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by recombinant murine GM-CSF following adsorption with anti-GM-CSF antiserum coupled on Sepharose 4B

Mating combination	Control mice, % resorbing	Treated mice†	
		Treatment	% Resorbing
CBA × DBA/2	42 (30, 22)	GM-CSF	14 (47, 8)*
		GM-CSF + antiserum	38 (29, 18)
		GM-CSF on control column	24 (41, 13)*
CBA × BALB/c	7 (36, 3)*		

†Killed on Day 14·5.
*$P < 0.05$ compared with CBA × DBA/2 control.

Discussion

The results presented in this paper clearly demonstrate that pregnancy outcome can be positively or negatively regulated by injecting lymphokines directly into pregnant females. This raises the question of the mechanisms involved in this regulation. The trophoblast is resistant to cell-mediated lysis by normal killer cells (cytotoxic T lymphocytes, CTLs) or natural killer cells (NK), but is sensitive to lysis by lymphokine-activated killer cells (LAKC)s or CTLs obtained from a mixed lymphocyte reaction performed in a culture medium (Opti-MEM, Gibco) (a derivative of Minimal Essential Medium (MEM)) which is optimized especially for LAKCs culture generation, and which yields from a mixed lymphocyte culture/reaction CTLs endowed with LAKC-like killing activity (Gendron & Baines, 1988; Drake & Head, 1989). The peri-implantation embryo is surrounded by NK cells (Croy *et al.*, 1987) and some non-lymphoid cells at the implantation site, such as granulated metrial gland cells, have some NK-like activity, exerting cytostatic effects on 3·5-day blastocysts and 6·5-day embryonic tissue of mice (Croy & Kassouf, 1989). It is tempting to speculate that they could themselves be regulated by lymphokines, and secrete some lymphokines, and thus be involved in the control of placental growth (Wegmann *et al.*, 1989.

Furthermore, it has been shown that spontaneous abortion rates in the abortion-prone CBA × DBA/2 mouse system correlate with the local infiltration of asialo GM1-positive cells (Gendron & Baines, 1988). Indeed, it has been shown that embryos are first infiltrated by cells with NK-like activity, and only thereafter by some cytotoxic T lymphocytes (Chaouat, 1986). Trophoblast cells themselves are not sensitive to NK cell lysis, but human and mouse embryonic

fibroblasts, for example, are. Activated NKs and LAKCs are therefore a probable threat to fetal survival. It ensues that the local T cell activation/expansion by interleukin-2 and interleukin-4, -5 and -6, the activation/enhancement of NK lytic activity, also mostly by these lymphokines, and the differentiation and expansion in the decidua of LAKCs needs to be down-regulated at the feto–maternal interface for fetal survival. It is achieved in the decidua by decidual suppressor cells that secrete a TGF-β-2-like molecule (Clark *et al.*, 1988) and a placenta-derived factor which has immunosuppressive activity coupled to fibroblast growth factor properties, and other TGF-β-2-like properties (Chaouat *et al.*, 1990). (It is worth recalling that TGF-β 2 can block the proliferation/activation of the aforementioned lymphocyte subsets and, in addition, exert direct protective effects on targets of products from activated T and NK cells, exactly as does TNF, when tested on such targets as murine embryonic fibroblasts, or the murine malignant fibroblastic cell line, L929.) Although placenta-mediated suppression is deficient in resorbing animals on Day 11 (Chaouat *et al.*, 1985), no such difference is seen at earlier stages, whereas there is a clear correlation between the level of decidual suppression at the implantation site and subsequent fetal resorption (Clark *et al.*, 1987). It is therefore possible that a defect in local suppression would result in too high a level of local NK-cell activity, and recruitment and/or activation of LAKCs, thus leading to embryo demise.

Environmental factors are involved in the CBA × DBA/2 resorption models (Hamilton & Hamilton, 1987), most likely microbial in nature and probably resulting in local activation of non-specific effectors. IL-2 can lead to NK-cell differentiation into LAKCs, and it is likely that injections of excess IL-2 by-pass local active suppression and trigger such detrimental activation/differentiation. Our data are in keeping with those of Tezabwala *et al.* (1989), although in our hands we need to inject higher doses than they do to obtain induction of abortion. Such discrepancy is most probably due to environmental conditions, as discussed below for 'basal' resorption rates.

NK activation can be directly achieved in pregnant females by Poly(I).Poly(C12)U injection. Injection of spleen cells from animals Poly(I).Poly(C12)U into CBA/J females, or direct treatment of these, elicits abortion (Baines & de Fougerolles, 1988; Chaouat *et al.*, 1988c, d, 1989a, b). Such effects are seen not only in CBA/J, but also in BALB/c, C57BL/6 mice, which are non-resorption-prone strains (Chaouat *et al.*, 1989b, c, 1990; unpublished data). This paper demonstrates that the effects on pregnancy outcome of such activated NK cell-enriched cellular transfer can be mimicked by injecting TNF and γ-interferon, which are products of such activation. Their action could be cytostatic for placental cells, as demonstrated *in vitro*, or directly cytotoxic for non-trophoblastic components of the placenta (such an effect is highly likely in the case of embryonic fibroblasts), whereas IL-2 is likely to act by activating LAKCs themselves. In addition, TNF could act by causing local necrosis as a consequence of its action on the blood vessels that penetrate the placenta.

TNF could be produced as a result of local triggering of decidual T cells and NK cells, as well as macrophages, as a bystander effect of infection. LPS would merely mimic the infection-related event. Such a mechanism would account for environmental or ageing effects in the CBA/J × DBA/2 system (Hamilton & Hamilton, 1987; Chaouat *et al.*, 1986, 1988b). We took advantage of such effects to enhance the basal resorption rates in our CBA/J mice by letting them age for the R GM-CSF experiments. This effect accounts for most of the variation one can observe between our tables, since mice of the same age in the same room had no gross (statistically significant) variations in resorption rates throughout the year.

The effect of IL-3 is more complicated to explain. Such a therapeutic effect does not necessarily represent a physiologically significant event, because IL-3 has not yet been demonstrated at the maternal–fetal interface, although one report describes the presence of IL-3 mRNA in the decidua (Shorter *et al.*, 1989). Nevertheless, placental cells appear to be responsive *in vitro* to all members of the CSF family tested so far, and GM-CSF itself has been shown to have direct trophic effects on outgrowth of pure ectoplacental cone trophoblast (Armstrong & Chaouat, 1989).

GM-CSF can also be released from the decidua. The doses of recombinant murine GM-CSF that we inject are far too low to act directly on placental cell growth in the mouse, as deduced from in-vitro titration curves. Furthermore, human GM-CSF protects against high resorption rates in the murine CBA × DBA/2 matings. This lymphokine has no direct effect on murine placental cell growth *in vitro*, and human GM-CSF does not sustain the growth or promote colony formation by mouse progenitor cells. The involvement on placental or decidual cells of a new type of CSF receptor, able to mediate activities of this lymphokine on non-lymphoid progenitor, with no species restriction, has been suggested (G. Vadas, unpublished data) but not yet proved. The demonstration of GM-CSF receptor on human trophoblast, possibly a new receptor, could clarify this issue (Uzumaki *et al.*, 1989). In the meantime, to explain these effects, we suggest at least four possible mechanisms, which are not necessarily mutually exclusive, and deserve consideration for future experimentation. (1) GM-CSF could trigger local release of CSF1 or more GM-CSF by macrophages or other cells leading to a direct or indirect trophic effect. (2) CSF1 might be released by placental cells themselves in response to low doses of GM-CSF in a paracrine loop, and high doses would result in a negative feed back. (3) Since some T cell lymphomas have been shown to secrete CSF1 (Pralloran *et al.*, 1989), and since HILDA/LIF, a product of CD4 and CD8 T cells has a direct trophic effect on extra somatic cells (Moreau *et al.*, 1988), the possibility that GM-CSF might have an effect on such a T cell subset is worthy of further examination. (4) GM-CSF is involved with interferon in natural suppressor mechanisms (Cleveland *et al.*, 1988), which share a certain number of properties with decidua and placenta-associated suppression, and IL-3 can abrogate LAKC activation/generation in the human (Gallagher *et al.*, 1988). Using the measurement of TNF-α to assess cytotoxicity of decidual supernatant on the L929 malignant murine fibroblast cell line, a classical test of TNF activity, we observed an inverse correlation in the CBA × DBA/2 system between TNF and GM-CSF titres (assessed as HCSF activity) in the decidua (Chaouat *et al.*, 1990). Although L929 is sensitive to TNF-α and -β, as well as the cytotoxic effects of other lymphokines, it is tempting to speculate that placental growth is under the control of decidual stop signals, amongst which TNF production by activated natural killer cells and lymphokine-activated killer cells, as well as granulated metrial gland cells, could be an element. Down-regulation by members of the CSF family of lymphokines of TNF production would result in enhanced placental growth, which would be functionally indistinguishable from a direct trophic effect, and may operate in parallel with it.

Whatever the mechanism(s) involved the results described in this paper indicate a direct influence of lymphokines on reproductive outcome *in vivo*.

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References

Armstrong, D.T.A. & Chaouat, G. (1989) Effects of lymphokines and immune complexes on murine placental cell growth in vitro. *Biol. Reprod.* **40**, 400–406.

Athanassakis, I., Bleackley, R.C., Paetkau, V., Guilbert, L., Barr, P.J. & Wegmann, T.G. (1987) The immunostimulatory effects of T cells and T cell lymphokines on murine fetally derived placental cells. *J. Immunol.* **138**, 37–44.

Baines, M. & de Fougerolles, R. (1988) Modulation of Natural Killer activity influences resorption rates in CBA × DBA/2 matings. *J. Reprod. Immunol.* **11**, 147–153.

Chaouat, G., Kiger, N. & Wegmann, T.G. (1983) Vaccination against spontaneous abortion in mice. *J. Reprod. Immunol.* **8**, 389–394.

Chaouat, G., Kolb, J.P., Kiger, N., Stanislawski, M. & Wegmann, T.G. (1985) Immunological concomitants of vaccination against abortion in mice. *J. Immunol.* **134**, 1594–1598.

Chaouat, G. (1986) Placental infiltration of resorbing CBA × DBA/2 embryos. *J. Reprod. Immunol., Suppl* **1**, 134, Abstr.

Chaouat, G., Lankar, D., Kolb, J.P. & Clark, D.A. (1987) 2 modeles d'avortements d'origine immunitaire chez la souris de laboratoire: mecanismes abortifs, modalites et mecanismes du traitement par l'immunisation contre un male relie ou non relie suivant les differences antigeniques pere mere. In *Immunologie de la Relation feto-maternelle* (Colloq. INSERM No. 154), pp. 243–255. Ed. G. Chaouat. INSERM, Paris.

Chaouat, G., Menu, E., Athanassakis, I. & Wegmann, T.G. (1988a) Maternal T Cells regulate placental size and fetal survival. *Regional Immunology* **1**, 143–148.

Chaouat, G., Clark, D.A. & Wegmann, T.G. (1988b) Genetics aspects of the CBA/J × DBA/2 J and B10 × B10.A models of murine spontaneous abortions and prevention of leukocyte immunisation. In *Early Pregnancy Loss. Mechanisms and Treatment*, pp. 89–105. Eds W. R. Allen, D. A. Clark, T. J. Gill III, J. F. Mowbray & W. R. Robertson. RCOG Press, London.

Chaouat, G., Menu, E., Bustany, P., Rebut-Bonneton, C. & Wegmann, T.G. (1988c) Role du placenta dans le maintien de l'allogreffe fetale. *Reprod., Nutr. Develop.* **28**, 1587–1588.

Chaouat, G., Menu, E., Wegmann, T.G., Clark, D.A., Minkowski, M., Dy, M. & Thang, M.N. (1988d) Explications actuelles du maintien de l'allogreffe fetale et hypothèses de travail sur le mécanisme d'effet des vaccins antiavortifs (Modèles murins). *Contraception Fertilité Sexualité* **17**, 57–59.

Chaouat, G., Szekeres-Bartho, J., Menu, E., Kinsky, R., Thang, M.N., Dy, M. & Minkowski, M. (1989b) Placental interactions with maternal immune system. In *Experimental Models in Obstetrics and Gynaecology*. Ed. C. Romanini. Parthenon Publishing Ltd, London. In Press.

Chaouat, G., Menu, E., Kinsky, R., Dy, M., Minkowski, M., Delage, G., Thang, M.N., Clark, D.A., Wegmann, T.G. & Szekeres-Bartho, J. (1989c) Lymphokines and non specific cellular lytic effectors at the feto maternal interface affect placental size and survival. In *Reproductive Immunology, 1989*. Elsevier, Amsterdam. In Press.

Chaouat, G., Menu, E., Szekeres-Bartho, J., Rebut-Bonneton, C., Bustany, P., Kinsky, R., Dy, M., Minkowski, M., Clark, D.A. & Wegmann, T.G. (1990) Immunological and endocrinological factors that contribute to successful pregnancy. In *Molecular and Cellular Immunology of the Feto-maternal Interface* (in press). Eds T. G. Wegmann & T. J. Gill III. Oxford University Press.

Clark, D.A., McDermott, M. & Sczewzuk, M.R. (1980) Impairment of host versus graft reaction in pregnant mice: (II) Selective suppression of Cytotoxic cell generation correlates with soluble suppressor activity and successful allogeneic pregnancy. *Cell. Immunol.* **52**, 106–118.

Clark, D.A., Kiger, N., Guennet, J.L. & Chaouat, G. (1987) Local active suppression and successful vaccination against spontaneous abortion in CBA/J mice. *J. Reprod. Immunol.* **10**, 79–86.

Clark, D.A., Falbo, M., Bruce-Rowley, R., Banwatt, D. & Stedronska-Clark, J. (1988) Active suppression of host versus graft reaction in pregnant mice. (IX) Soluble suppressor activity obtained from allopregnant mouse decidua that blocks the cytolytic effector response is related to TGF Beta. *J. Immunol.* **141**, 3833–3841.

Clark, D.A., Head, J.R., Drake, B., Fulop, G., Brierley, J., Manuel, J., Barwatt, D. & Chaouat, G. (1990) Role of a factor related to transforming growth factor beta 2 in successful pregnancy. In *Molecular and Cellular Immunology of the Feto-maternal Interface* (in press). Eds T. G. Wegmann & T. J. Gill III. Oxford University Press.

Cleveland, M.G., Lane, R.G. & Klimpel, G.R. (1988) Spontaneous Interferon Beta production. A common feature of Natural Suppressor Systems. *J. Immunol.* **141**, 2043–2048.

Croy, B.A. & Kassouf, S.A. (1989) Evaluation of the murine metrial gland for immunological function. *J. Reprod. Immunol.* **15**, 51–71.

Croy, B.A., Wood, W. & King, G.J. (1987) Evaluation of intrauterine immune suppression during pregnancy in a species with epitheliochorial placentation. *J. Immunol.* **139**, 1088–1095.

Drake, B.L. & Head, J.R. (1988) Murine trophoblast cells are susceptible to Lymphokine Activated killer (LAK) cell lysis. *Am. J. Reprod. Immunol.* **16**, 114, Abstr.

Drake, B.L. & Head, J.R. (1989) Murine trophoblast cells can be killed by allospecific cytototoxic T lymphocytes generated in Gibco OPTI MEM medium. *J. Reprod. Immunol.* **15**, 71–77.

Dy, M., Schneider, E., Gastinel, L.N., Aufray, C., Mermod, J.J. & Hamburger, J. (1987) Histamine Colony Stimulating Factor (HCSF) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) activities are borne by the same molecule. *Eur. J. Immunol.* **17**, 1243–1247.

Gallagher, G., Wilcox, F. & Al-Azzawi, F. (1988) Interleukin 3 and Interleukin 4 each strongly suppress induction and function of human LAK cells. *Clin. exp. Immunol.* **74**, 166–171.

Gendron, R.L. & Baines, M. (1988) Infiltrating decidual Natural Killer cells are associated with spontaneous abortion in mice. *Cell. Immunol.* **113**, 261–265.

Gendron, R.L., Nestel, F.P., Lapp, W.S. & Baines, M.G. (1989) Lipopolysaccharide induced fetal resorption involves embryo associated production of Tumor Necrosis Factor. *Proc. 7th Int. Congr. Immunol., Berlin*, Abstr. 118-017.

Hamilton, M.S. & Hamilton, B.L. (1987) Environmental influences on immunologically associated recurrent spontaneous abortion in CBA/J mice. *J. Reprod. Immunol.* **11**, 237–241.

Mowbray, J.F., Lidell, H., Underwood, J.L., Gibins, S.C., Reginald, P.W. & Beard, R.W. (1985) Controlled trial of treatment of recurrent spontaneous abortions by immunisation with paternal cells. *Lancet* **i**, 941–943.

Parand, M. (1987) Role of TNF in non specific stimulation of mouse resistance to infection. *Immunobiology* **175** (1/2), 26, Abstr.

Parand, M. & Chedid, L. (1964) Protective effects of chlorpromazine against endotoxin induced abortion. *Proc. Soc. exp. Biol. Med.* **116**, 906–915.

Moreau, J.F., Donaldson, D.D., Bennet, F., Witek Giannotti, J., Clark, S.C. & Wong, G.G. (1988) Leukemia inhibitory factor is identical to the myeloid growth factor for human interleukin for DA cells. *Nature, Lond.* **336**, 6200, 690–692.

Pralloran, V., Gascan, H., Boursier, M.C. & Soulillou, J.P. (1989) Sécrétion de M-CSF/CSF/1 par un lymphome T. *Proc. French Soc. Immunol., Nantes* p. 49, Abstr.

Shorter, S.C., Vince, G.S. & Starkey, P.M. (1989) The identification of mRNA for the IL-3 related cytokines in placental and decidual tissues. *Proc. 3rd Mtg Eur. Placenta Group.* Eds L. Cedard, G. Chaouat, J. C. Challier & C. Neismann. INSERM, Paris. In press.

Taylor, C. & Faulk, W.P. (1981) Prevention of recurrent spontaneous abortions with leukocyte transfusion. *Lancet* ii, 68–70.

Tezabwala, B.U., Johnson, P.M. & Rees, R.C. (1989) Inhibition of pregnancy viability in mice following IL-2 administration. *Immunology* **67**, 115–120.

Uzumaki, H., Okabe, T., Sasaki, N., Hagiwara, K., Takaku, F., Tobita, M., Yasukawa, K., Ito, S. & Umezawa, Y. (1989) Identification and characterisation of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. *Proc. natn. Acad. Sci. USA.* **86**, 9323–9326.

Wegmann, T.G., Athanassakis, I., Guilbert, L., Branch, D., Dy, M., Menu, E. & Chaouat, G. (1989) The role of M-CSF and GM-CSF in fostering placental growth, fetal growth, and fetal survival. *Transplantation Proc.* **21**, 566–569.

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Effects of granulosa cell co-culture on in-vitro meiotic resumption of bovine oocytes

M. A. Sirard and S. Bilodeau

Département de Zootechnie, Université Laval, Québec, Canada G1K 7P4

Summary. This study was undertaken to create an in-vitro model using granulosa cell monolayers to replace the role of the follicle in the maturation of bovine oocytes. Cumulus–oocyte complexes were co-incubated with fresh or 7-day granulosa cell cultures (with new or conditioned medium) or with conditioned medium alone, in the presence or absence of IBMX (isobutylmethylxanthine), adenosine or heparin. Progression to the metaphase-II stage was significantly affected by the co-culture of oocytes with bovine granulosa cell monolayers and to a lesser degree when cultured with supernatant alone (conditioned medium). The oocytes attached rapidly to the monolayer, suggesting that the intimate contact between the granulosa cells and the cumulus–oocyte complexes is an important signal for the maintenance of meiotic arrest. Heparin did not prevent maturation itself, but prevented attachment of cumulus–oocyte complexes to monolayers, thereby reducing their inhibitory effect. Adenosine prevented cumulus expansion and reduced maturation and IBMX was an effective inhibitor only in the presence of additional granulosa cells.

Keywords: oocyte; meiosis; granulosa cells; *in vitro*; cattle

Introduction

Pincus & Enzmann (1935) showed that mammalian oocytes, once removed from the precise environment of the follicle, can resume the first meiotic division spontaneously in simple culture media. For cattle, information on the meiotic process is needed to enhance the developmental potential of in-vitro matured oocytes, since one possible cause of developmental defects is the incompetence of oocytes from the smaller follices. If germinal vesicle arrest could be maintained *in vitro*, it would be possible to influence both the cytoplasm and the nucleus with hormones or granulosa cells before nuclear maturation.

Bovine oocytes are not as sensitive as oocytes from other animals studied to protein kinase stimulation via cAMP accumulation (Sirard & First, 1988). Results obtained for cattle (Sirard & First, 1988) and sheep (Moor & Heslop, 1981) indicate that cAMP accumulation in the oocyte may not be the only physiological way to maintain the meiotic arrest in spite of the fact that bovine oocytes possess an active adenylate cyclase enzyme (Kuyt *et al.*, 1988) and respond temporarily to cAMP variations (Homa, 1988; Sirard & First, 1988). The effect of isobutylmethylxanthine (IBMX) in cows is similar to the effect of cAMP analogues and results in a delayed breakdown of the germinal vesicle (GVBD) followed by normal maturation or a metaphase-I block (Ball *et al.*, 1984; Sirard & First, 1988). If denuded oocytes are used, much greater amounts (5 mM, 25 times) of IBMX are required to inhibit GVBD (Homa, 1988). In the mouse, cAMP variations are modulated principally by phosphodiesterase inhibitors, and purines are therefore also effective inhibitors since their major effect is related to the inhibition of phosphodiesterase (Downs *et al.*, 1989). Purines have a weak effect on the meiotic resumption of bovine oocytes (Sirard & First, 1988) and since bovine follicular fluid does not contain amounts of purines comparable to those

IN THE MATTER OF
US Patent Application No. 09/380,327
by Robertson et al

EXHIBIT DAC-4

This is Exhibit DAC-4 referred to in the Statutory Declaration dated 2 July 2004 by David Alexander Clark.

David A. Clark

Before me:

July 2, 2004
Burlington, Ontario

A person empowered to witness Statutory Declarations under the laws of the Province of Ontario, Canada

FULVIO DERIDATO
Notary Public.
etc.

Identification of gonadotropin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. *Biol. Reprod.* 37, 1075–1082.

Fowler, P.A., Messinis, I.E. & Templeton, A.A. (1989) Gonadotrophin surge-attenuating factor (GnSAF) activity in human follicular fluid is different from inhibin. *J. Reprod. Fert., Abstr. Ser.* No. 4, 30, abstr.

Fraser, H.M. & Lunn, S.F. (1989) Rapid inhibitory effects of an LHRH agonist implant on the oestrogen-induced LH surge and the induction of a defective luteal phase after an agonist-induced ovulation in the macaque. *Hum. Reprod.* 4, 506–512.

Fraser, H.M., Abbott, M., Laird, N.C., McNeilly, A.S., Nestor, J.J., Jr & Vickery, B.H. (1986) Effects of an LH-releasing hormone antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (*Macaca arctoides*). *J. Endocr.* 111, 83–90.

Fraser, H.M., Sandow, J., Seidel, H. & von Rechenberg, W. (1987) An implant of a gonadotropin releasing hormone agonist (buserelin) which suppresses ovarian function in the macaque for 3–5 months. *Acta endocr., Copenh.* 115, 521–527.

Fraser, H.M., Robertson, D.M. & de Kretser, D.M. (1989) Immunoreactive inhibin concentrations in serum throughout the menstrual cycle of the macaque: suppression of inhibin during the luteal phase by treatment with an LHRH antagonist. *J. Endocr.* 121, R9–R12.

Glasier, A., Thatcher, S.S., Wickings, E.J., Hillier, S.G. & Baird, D.T. (1988) Superovulation with exogenous gonadotropins does not inhibit the luteinizing hormone surge. *Fert. Steril.* 49, 81–85.

Land, R.B., Morris, B.A., Baxter, G., Fordyce, M. & Forster, J. (1982) Improvement of sheep fecundity by treatment with antisera to gonadal steroids. *J. Reprod. Fert.* 66, 625–634.

Littman, B.A. & Hodgen, G.D. (1984) Human menopausal gonadotropin stimulation in monkeys: blockade of the luteinizing hormone surge by a highly transient ovarian factor. *Fert. Steril.* 41, 440–447.

McLachlan, R.I., Robertson, D.M., Healy, D.L., Burger, H.G. & de Kretser, D.M. (1987) Circulating immunoreactive inhibin levels during the normal human menstrual cycle. *J. clin. Endocr. Metab.* 65, 954–961.

McLachlan, R.I., Robertson, D.M., Healy, D.L., de Kretser, D.M. & Burger, H.G. (1986) Plasma inhibin levels during gonadotrophin-induced ovarian hyperstimulation for IVF: a new index of follicular function? *Lancet*, 1233–1234.

Messinis, I.E. & Templeton, A.A. (1987) Endocrine and follicle characteristics of cycles with and without endogenous luteinizing hormone surges during superovulation induction with pulsatile follicle-stimulating hormone. *Hum. Reprod.* 2, 11–16.

Messinis, I.E. & Templeton, A.A. (1989) Pituitary response to exogenous LHRH in superovulated women. *J. Reprod. Fert.* 87, 633–639.

Robertson, D.M., Giacometti, M., Foulds, M., Lahnstein, J., Goss, N.H., Hearn, M.T.W. & de Kretser, D.M. (1989) Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. *Endocrinology* 125, 2141–2149.

Robertson, D.M., Tsonis, C.G., McLachlan, R.I., Handelsman, D.L., Leask, R., Baird, D.T., McNeilly, A.S., Hayward, S., Healy, D.L., Findlay, J.K., Burger, H.G. & de Kretser, D.M. (1988a) Comparison of inhibin immunological and in vitro biological activities in human serum. *J. clin. Endocr. Metab.* 119, 438–443.

Robertson, D.M., Hayward, S., Irby, D., Jacobsen, J., Clarke, L., McLachlan, R.I. & de Kretser, D.M. (1988b) Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. *Molec. cell. Endocrinol.* 58, 1–8.

Schenken, R.S. & Hodgen, G.D. (1983) Follicle stimulating hormone induced ovarian hyperstimulation in monkeys: blockade of the luteinizing hormone surge. *J. clin. Endocr. Metab.* 57, 50–55.

Stillman, R.J., Williams, R.F., Lynch, A. & Hodgen, G.D. (1983) Selective inhibition of follicle stimulating hormone by porcine follicular fluid extracts in the monkey: effects on midcycle surges and pulsatile secretion. *Fert. Steril.* 40, 823–828.

Tsonis, C.G., Messinis, I.E., Templeton, A.A., McNeilly, A.S. & Baird, D.T. (1988) Gonadotropic stimulation of inhibin secretion by the human ovary during the follicular and early luteal phase of the cycle. *J. clin. Endocr. Metab.* 66, 915–921.

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Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy

G. Chaouat*, E. Menu*, D. A. Clark†, M. Dy‡, M. Minkowski‡ and T. G. Wegmann§

**U 262 INSERM, Clinique Universitaire Baudelocque, 123 Bd de Port Royal, 75674 Paris Cedex 14, France; †McMaster University, Department of Medicine, Hamilton, Ontario, Canada L8N 3Z1; ‡U 25 INSERM, Hopital Necker, 149 rue de Sèvres, 75015 Paris, France; and §Department of Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7*

Summary. In this study, we examined the effect of injecting various cytokines. We report here that tumour necrosis factor (TNF)α, γ-interferon and interleukin 2 (IL-2) can, in some circumstances, increase fetal resorption rates in abortion-prone (CBA/J × DBA/2) and non-abortion prone (CBA/J × BALB/c,C3H × DBA/2) matings: 1000 units TNF enhanced resorptions from 43 to 79% in CBA × DBA/2, from 7 to 89% in CBA × BALB/c, from 5 to 47% in C3H × DBA/2. The effect was both gestational age- and dose-dependent. Gamma interferon and R-IL-2 enhanced resorptions from 38 to 68% and 76% respectively in the CBA/J × DBA/2 mating combination, whereas the rates in CBA/J × BALB/c matings were enhanced from 6 to 44% and 55%. Lipopolysaccharide (LPS), which is known to lead to the release of TNF-α, had a similar effect, leading to gestational age- and dose-dependent enhancement of resorptions up to 100%.

However, cytokines of the CSF family, including IL-3 and GM-CSF, increased the chances of fetal survival when injected into abortion-prone mice, e.g. reducing resorption rates in the abortion-prone CBA/J × DBA/2 mating combination from 55 to 22% (IL-3), and 47 to 8% (GM-CSF). They also increased fetal and placental weight and, in particular, expanded the spongiotrophoblast zone in the placenta. The latter observations may be due to a direct trophic influence on placental cells, perhaps through a cytokine cascade, or an indirect effect due to inhibition of natural killer (NK)-like cells, or both. Whatever the mechanism, these results may find practical application in influencing reproductive outcome in women and other species.

Keywords: abortion; mice; lymphokines; induction; treatment

Introduction

There is increasing evidence that some recurrent abortions in women might be preventable by immunization of the mother against paternal or third party alloantigens (Taylor & Faulk, 1981; Mowbray *et al.*, 1985). Ethical concerns limit the extent of studies on this subject in women but there now exist several models of pregnancy failure in mice and its correction by lymphocyte alloimmunization.

The best studied model is the mating of CBA females with DBA/2 males: CBA/J, but not C3H-HeJ, females have an abnormal resorption rate when mated with DBA/2 males (Clark *et al.*, 1980), but when CBA/J females are mated with BALB/c males, the fetal resorption rate is normal (Chaouat *et al.*, 1983). In this system, there is suggestive evidence that abortion is mediated by asialo GM1-positive natural effector cells (Chaouat *et al.*, 1987; Baines & de Fougerolles, 1988). If

CBA/J females are immunized against BALB/c lymphocytes before mating, the high resorption rates in CBA/J mice mated with DBA/2 males are prevented (Chaouat *et al.*, 1983; 1987, 1988a, b, c) and there is no longer accumulation of asialo Gm1+ cells in the uterus (Gendron & Baines, 1988). Protection against abortion can be adoptively transferred by CBA/J anti-BALB/c immune T cells, or T cells from the spleens of CBA/J mice that were multiparous by repeated mating to BALB/c males (Chaouat *et al.*, 1985). Furthermore, T cell-derived cytokines such as IL-3, GM-CSF and CSF-1 were strongly supportive of the in-vitro proliferation of placental cells (Athanassakis *et al.*, 1987; Armstrong & Chaouat, 1989), and, conversely, tumour necrosis factor (TNF) -α and -β proved to be cytostatic for these placental cells *in vitro*. In addition, in-vivo depletion of T cells enhanced resorption rates in CBA females mated with DBA/2 males as well as in the non-abortion prone CBA × BALB/c mating (Chaouat *et al.*, 1988a). Further support for a trophic role of activated T cells *in vivo* came from the observation that depletion of T cells in the autoimmune MRL lpr/lpr strain corrected placental hypertrophy and placental phagocytic activity was then normal. This observation was corroborated by the finding that transfer of cells from MRL lpr/lpr mice into H-2k-compatible CBA/J females prevented the CBA × DBA/2 pregnancy from showing increased fetal resorption (Chaouat *et al.*, 1988a, b). Since these mice are known to produce excess IL-3, and autoantibodies with IL-3-like activity, and based on the aforementioned evidence, we decided to test directly various lymphokines, all of them products from activated T cells, for their effect on fetal resorption in CBA/J mice mated with DBA/2 males.

Materials and Methods

Mice. CBA/J mice were obtained from Iffa Credo (L'Arbresle, France) at the age of 8 weeks. They were kept for another 2 weeks in our animal facility before mating. The day of observing a vaginal plug was termed Day 0·5 of pregnancy.

Since the rate of resorption increases with ageing or environmental conditions (Chaouat *et al.*, 1987, 1988a; Hamilton & Hamilton, 1987) mice were in some cases aged a further 5 weeks before mating in a conventional room where abortions were known to be higher (Chaouat *et al.*, 1987, 1988a) than in the gnotobiotic facility. C3H/HeJ, C57BL/6, DBA/2 and BALB/cJ mice were obtained from the IRSC, Villejuif, France, at the age of 8 weeks, and also maintained for 2 weeks before use.

For experiments performed in Canada 6–8-week-old CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained until 12 weeks of age when they were mated.

Fetal resorptions were assessed on Day 14·5 of pregnancy, unless otherwise stated. In some experiments, animals were allowed to deliver to confirm the anti-abortive effect by direct assessment of viable offspring. This technique suffers from the fact that some pregnant mothers eat their offspring and placentae immediately after birth: the mean litter size was therefore determined on Day 1, as the 'surviving' litter size. Animals which had clearly eaten their progeny were excluded from the experiment. There was no indication that any treatment which we employed had an influence on this type of behaviour.

Lymphokines and reagents. Purified GM CSF was obtained from the P338 D1 cell line as described by Dy *et al.* (1987). Recombinant murine GM-CSF was purchased from Genzyme Inc. (Boston, MA, USA) directly or via Tebu France (Le Perray, Yvelines, France). Recombinant human GM-CSF (produced in CHO cells) was a kind gift from the Genetics Institute, Boston, MA, USA. All recombinant materials were purified to homogeneity by the manufacturers, and did not contain other lymphokines or lipopolysaccharide (LPS) that might interfere with the assay. Lipopolysaccharide (Difco, via Sigma France, itself via OSI France, Paris, France) extracted from *Salmonella enteritidis* was from a batch pretested in various LPS and LPS/TNF assays, and was a kind gift of Dr M. Parand, Institut Biomédical des Cordeliers, Paris, France. Recombinant human TNF-α, murine and human IL-2, and murine γ-interferon were kindly donated by Dr J. Wietzerbin, Institut Curie, Paris, France. Rabbit polyclonal antibody against murine GM-CSF was kindly donated by the Glaxo Institute for Molecular Biology, Geneva, Switzerland. Purified IL-3 was prepared from the WEHI cell line as described by Dy *et al.* (1987).

LPS was injected i.v. at 1 μg on Day 7 or 12, or 3 μg on Day 15. Mice were killed on Day 12 when injection was performed on Day 7, on Day 16 for injection performed on Day 12, or Day 19 for injection performed on Day 15. For TNF experiments, each group was injected i.v. with 1000 units of human recombinant TNF-α or saline on Day 7·5 of gestation, and mice were examined on Day 14·5 for fetal resorption. Alternatively, 1000, 2000 or 3000 units TNF were injected on various days of gestation and mice were checked on Day 12 (injections until Day 10·5) or 17 (injection on Day 13·5) or 19·5 (just before parturition) for injection on Day 17·5. Gamma interferon was given at 1500 units i.p. on Day 7·5 of gestation, and R-IL-2 was given at 2000 units i.p. on Days 6·5, 8·5 and 10·5 of gestation. Mice were killed on Day 14·5. Other mice received 600 units IL-3 i.p. on Day 6·5, 8·5 or 10·5 and were killed on Day 14·5.

GM-CSF was injected i.p. as (a) 400 HCSF units in 0·2 ml saline on Days 6·5, 8·5 or 10·5 or (b) 200 HCSF units in phosphate-buffered saline (PBS) on Day 6·5, 8·5 or 10·5.

For dose–response experiments 200, 400, 1000 or 2500 units of GM-CSF were injected i.p. in PBS. In some cases, GM-CSF was dissolved in medium RPMI 1640 before injection. For the experiments that were conducted in Canada with recombinant human GM-CSF (Genetic Institute) and murine GM-CSF (Genzyme), a single dose of 200 units was injected on Day 7·5 of pregnancy.

Spongiotrophoblast width. To estimate spongiotrophoblast width (Table 7), spongiotrophoblasts were sliced from fresh placentae with a surgical lance, then snap-frozen and measured by calipers in mm. The labyrinthine zone was also measured but the enlargement was not statistically significant in the limits of these experiments (number of placentae studied) and the data are therefore not shown.

Statistics. Student's *t* tests (placental width, fetal and placental weights) and χ^2 analysis (% resorptions) were performed to test the significance of treatment effects.

Results

Negative effects on pregnancy outcome

LPS from *Salmonella enteritidis* has been shown to induce complete fetal death in several strains of mice at a dose of 1 μg per mouse when given i.v. on Day 7·5 or Day 12·5 (a dose of 3 μg per mouse is required for abortion on Day 15–18) (Parand & Chedid, 1964), irrespective of the strain.

Table 1 shows typical experiments that confirm these data for various mating combinations of mice which are not abortion prone, as well as in the CBA × DBA/2 abortion-prone mice.

Direct measurement of TNF-α in the decidua confirmed that abortion was correlated with an enhancement of TNF levels (data not shown, and Chaouat *et al.*, 1990), in agreement with previous data (Parand & Chedid, 1964; Parand, 1987; Gendron *et al.*, 1989).

Table 1. Induction of fetal resorption (%, with no. viable, no. resorbing in parentheses) by lipopolysaccharide in mice of various mating combinations injected on Day 7, 12 or 15

Mating combination	Control mice	Treated mice*		
		1 μg i.v.		3 μg i.v.
		Day 7	Day 12	Day 15
C3H × BALB/c	6 (43, 2)	98 (1, 45)	100 (0, 48)	91 (4, 41)
C57BL/6 × DBA/2	6 (44, 3)	96 (1, 42)	98 (1, 39)	95 (2, 40)
C57BL/6 × BALB/c	8 (47, 4)	100 (0, 48)	96 (2, 53)	98 (1, 50)
CBA/J × BALB/c	10 (45, 4)	100 (0, 51)	100 (0, 48)	92 (4, 48)
CBA/J × DBA/2	38 (17, 28)	100 (0, 45)	100 (0, 49)	100 (0, 43)
C3H × DBA/2	5 (39, 2)	100 (0, 39)	100 (0, 40)	100 (0, 45)

*Mice were killed on Day 12 (injection on Day 7), Day 16 (injection on Day 12) or Day 19 (injection on Day 15).

All treatment groups showed significantly more resorptions than in the respective control groups, $P < 0.001$ (χ^2 tests).

When 1000 units of recombinant human TNF-α were injected on Day 7·5 i.p., a significant increase in fetal resorption rates was observed in CBA × DBA/2 mice as well as in the non-abortion-prone CBA × BALB/c and C3H × DBA/2 combinations. The effect was still significant when TNF-α was given on Days 9 and 10. However, to obtain a comparable level of fetal resorption rate, a higher dose of TNF was required on Days 13 and 17, although 1000 units significantly enhanced the fetal resorption rates above background levels (Table 2).

Poly(I).Poly(C12U), an inducer of γ-interferon production and an activator of natural killer cells (NKs) and lymphokine-activated killer cells (LAKCs), is known to increase fetal resorption rates (Baines & de Fougerolles, 1988; Chaouat *et al.*, 1988a, 1989a, b, c; R. Kinsky, G. Lepage,

Table 2. Induction of fetal resorptions (no. viable, no. resorbing) in mice by human recombinant TNF-α

Exp.	Mating combination	Control mice, % resorptions	Treated					
			Days of injection	% Resorption	Days of injection	% Resorption	Days of injection	% Resorption
1 (1000 Units)†	CBA × DBA/2	43 (12, 19)	7·5	79 (6, 22)*	—	—	—	—
	CBA × BALB/c	7 (26, 2)	7·5	89 (5, 39)*	—	—	—	—
	C3H × DBA/2	5 (34, 2)	7·5	47 (15, 17)*	—	—	—	—
2 (1000 Units)‡	CBA × DBA/2	36 (39, 22)	0·5	31 (43, 19)	3·5	83 (6, 30)*	—	—
			5·5	67 (22, 45)*	7·5	83 (11, 55)*	—	—
			9·5	88 (8, 58)*	10·5	75 (14, 43)*	—	—
			13·5	52 (35, 38)	17·5	47 (30, 27)	—	—
3	CBA × DBA/2	39 (33, 21)						
1000 Units†			7·5	89 (5, 41)*	13·5	60 (18, 26)*	17·5	51 (28, 29)
2000 Units†			7·5	100 (0, 42)*	13·5	94 (3, 45)*	17·5	57 (22, 29)
3000 Units†			7·5	100 (0, 48)*	13·5	59 (25, 36)*	17·5	85 (8, 45)*

†Killed Day 14·5.
‡Killed Day 12 (injections on Days 0·5–10·5), Day 17 (injections on Day 13·5) or Day 19·5 (injections on Day 17·5).
*$P < 0·05$ compared with control value for that Exp.

M. N. Thang & G. Chaouat, unpublished). We therefore also tested the effects of treatment with γ-interferon, and recombinant murine IL-2, a LAKCs and NK cell activator. Both treatments enhanced fetal resorption in the CBA × DBA/2 and CBA × BALB/c mating combinations (Table 3).

Table 3. Enhancement of fetal resorptions (%, no. viable, no. resorbing) by murine recombinant γ-interferon (1500 Units on Day 7·5) and murine recombinant IL-2 (2000 Units on Days 6·5, 8·5 and 10·5)

Mating combination	Control mice	Treated mice†	
		γ-Interferon	IL-2
CBA/J × DBA/2	38% (18, 11)	68% (8, 17)*	76% (8, 17)*
CBA/J × BALB/c	6% (31, 2)	44% (18, 14)*	55% (15/18)*

†Killed on Day 14.
*$P < 0·05$ compared with control.

Positive effects on pregnancy outcome

When IL-3 was injected i.p. fetal resorption rates were decreased in CBA × DBA/2 pregnancies whereas in the same experiment treatment with LPS or recombinant human TNF had the opposite effect (Table 4).

Table 4. Reduction of CBA × DBA/2 fetal resorption by murine IL-3 from WEHI cells

Exp.	Control mice			Treated mice†			
	% Resorbing	Placental wt (mg)	Fetal wt (mg)	Treatment	% Resorbing	Placental wt (mg)	Fetal wt (mg)
1	52 (14, 13)	102	121	IL-3‡	28 (15, 6)*	125*	141*
2	55 (17, 21)	—	—	IL-3†	22 (25, 7)*	—	—
3	46 (22, 19)	—	—	IL-3†	19 (30, 7)*	—	—
				LPS§	88 (3, 22)*	—	—
				H-rTNF-α¶	93 (2, 27)*	—	—
				PBS	54 (18, 21)	—	—

†Killed on Day 14·5.
‡600 Units on Days 6·5, 8·5 and 10·5.
§0·1 μg in PBS on Days 6·5, 8·5 and 10·5.
¶500 Units on Days 6·5, 8·5 and 10·5.
*$P < 0·05$ compared with controls.

Treatment with 200 or 400 units of purified natural GM-CSF decreased CBA × DBA/2 fetal resorption rates (Table 5). When different doses of GM-CSF were tested, dosages of > 1000 Units per injection were ineffective (Table 5). In fact, 3 × 2500 HCSF units sometimes enhanced fetal resorptions, although most often the enhancement was not significant, and at best P values were $<0·02$ (data not shown).

Treatment with recombinant murine GM-CSF (Genzyme) given on the same schedule at low doses was also protective (Table 6).

Both forms of murine GM-CSF not only affected fetal survival, but also enhanced placental (and fetal) weights (Tables 5 and 6). The enhancement appeared to depend markedly upon additional spongiotrophoblast width as a result of treatment (see Table 6). No significant size enhancement was traced in the labyrinth (data not shown).

Table 5. Reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by purified murine GM-CSF from P338 D1

Exp.	Mating combination	Control mice: % Resorbing	Control mice: Placental wt (mg)	Control mice: Fetal wt (mg)	Treated mice† Treatment‡	Treated mice†: % Resorbing	Treated mice†: Placental wt (mg)	Treated mice†: Fetal wt (mg)
1	CBA × DBA/2	47 (27, 24)	95	118	GM-CSF400	8 (44, 4)*	136*	209*
	CBA × BALB/c	8 (37, 3)	138	211				
2	CBA × DBA/2	37 (17, 10)	—	—	PBS	51 (15, 6)	—	—
					GM-CSF200	7 (29, 2)*	—	—
3	CBA × DBA/2	38 (26, 16)	—	—	GM-CSF200	17 (38, 8)*	—	—
					GM-CSF400	24 (32, 10)*	—	—
					GM-CSF1000	32 (27, 13)	—	—
					GM-CSF2500	53 (18, 20)	—	—

†Killed on Day 14·5.
‡GM-CSF injected on Days 6·5, 8·5 and 10·5 at dose indicated.
*$P < 0.05$ compared with controls.

Table 6. Reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by recombinant murine and human GM-CSF

Exp.	Mating combination	Control mice: % Resorbing	Control mice: Plac. wt (mg)	Control mice: Fetal wt (mg)	Control mice: Spongiotrophoblast width (mm × 10^{-1})	Treated mice† Treatment‡	Treated mice†: % Resorbing	Treated mice†: Plac. wt (mg)	Treated mice†: Fetal wt (mg)	Treated mice†: Spongiotrophoblast width (mm × 10^{-1})
1	CBA × DBA/2	52 (14, 13)	102	122	24·8	GM-CSF from P338 D1	24 (41, 13)*	140*	205*	33·5*
						R murine GM-CSF	30 (33, 14)*	133*	200*	33·1*
	CBA × BALB/c					R murine GM-CSF	17 (24, 5)*	141*	193*	30·6*
2	CBA × DBA/2	66 (11, 21)	—	—	—	R murine GM-CSF	10 (18, 2)*	—	—	—
	CBA × BALB/c	18 (29, 6)	—	—	—					
3	CBA × DBA/2	29 (43, 150)	—	—	—	Human R GM-CSF§	18 (32, 150)*	—	—	—
						R murine GM-CSF§	21 (51, 247)*	—	—	—

In Exps 1 and 2 mice were aged (see 'Methods'). Experiment 3 was carried out in Canada.
†Killed on Day 14·5.
‡On Days 6·5, 8·5 and 10·5.
§On Day 7·5.
*$P < 0.05$ compared with controls.

Similar experiments conducted in Canada used a recombinant human GM-CSF (Genetic Institute) and the same murine GM-CSF (Genzyme), but the control levels of resorption were much lower. A single dose of 200 units of either form of GM-CSF on Day 7·5 of pregnancy was minimally effective (Table 6). The murine GM-CSF stimulated placental cell growth *in vitro* but the human recombinant GM-CSF was inactive in this regard (data not shown, and Clark *et al.*, 1990). High doses of the human GM-CSF (10 000–30 000 units/mouse) were ineffective, similar to the data shown in Table 5.

Since P 338 D1 purified material contained traces of IL-1 and IL-3, it was important to ascertain that the effects of P 338 D1 supernatant and recombinant GM-CSF were mediated by GM-CSF itself and not by some contaminant in the preparation. Recombinant murine GM-CSF, or P 338 D1 material, was passed through a column containing normal rabbit serum coupled to CNBr-activated Sepharose 4B (Pharmacia, Pharmacia-LKB, Les Ulis, France) or similarly prepared rabbit anti-murine GM-CSF. The specific antiserum retained the fetal protective effect, while the control column eluant did not (Table 7).

Table 7. Removal of reduction of CBA × DBA/2 fetal resorption (no. viable, no. resorbing) by recombinant murine GM-CSF following adsorption with anti-GM-CSF antiserum coupled on Sepharose 4B

Mating combination	Control mice, % resorbing	Treated mice†	
		Treatment	% Resorbing
CBA × DBA/2	42 (30, 22)	GM-CSF	14 (47, 8)*
		GM-CSF + antiserum	38 (29, 18)
		GM-CSF on control column	24 (41, 13)*
CBA × BALB/c	7 (36, 3)*		

†Killed on Day 14·5.
*$P < 0.05$ compared with CBA × DBA/2 control.

Discussion

The results presented in this paper clearly demonstrate that pregnancy outcome can be positively or negatively regulated by injecting lymphokines directly into pregnant females. This raises the question of the mechanisms involved in this regulation. The trophoblast is resistant to cell-mediated lysis by normal killer cells (cytotoxic T lymphocytes, CTLs) or natural killer cells (NK), but is sensitive to lysis by lymphokine-activated killer cells (LAKC)s or CTLs obtained from a mixed lymphocyte reaction performed in a culture medium (Opti-MEM, Gibco) (a derivative of Minimal Essential Medium (MEM)) which is optimized especially for LAKCs culture generation, and which yields from a mixed lymphocyte culture/reaction CTLs endowed with LAKC-like killing activity (Gendron & Baines, 1988; Drake & Head, 1989). The peri-implantation embryo is surrounded by NK cells (Croy *et al.*, 1987) and some non-lymphoid cells at the implantation site, such as granulated metrial gland cells, have some NK-like activity, exerting cytostatic effects on 3·5-day blastocysts and 6·5-day embryonic tissue of mice (Croy & Kassouf, 1989). It is tempting to speculate that they could themselves be regulated by lymphokines, and secrete some lymphokines, and thus be involved in the control of placental growth (Wegmann *et al.*, 1989.

Furthermore, it has been shown that spontaneous abortion rates in the abortion-prone CBA × DBA/2 mouse system correlate with the local infiltration of asialo GM1-positive cells (Gendron & Baines, 1988). Indeed, it has been shown that embryos are first infiltrated by cells with NK-like activity, and only thereafter by some cytotoxic T lymphocytes (Chaouat, 1986). Trophoblast cells themselves are not sensitive to NK cell lysis, but human and mouse embryonic

fibroblasts, for example, are. Activated NKs and LAKCs are therefore a probable threat to fetal survival. It ensues that the local T cell activation/expansion by interleukin-2 and interleukin-4, -5 and -6, the activation/enhancement of NK lytic activity, also mostly by these lymphokines, and the differentiation and expansion in the decidua of LAKCs needs to be down-regulated at the feto–maternal interface for fetal survival. It is achieved in the decidua by decidual suppressor cells that secrete a TGF-β-2-like molecule (Clark *et al.*, 1988) and a placenta-derived factor which has immunosuppressive activity coupled to fibroblast growth factor properties, and other TGF-β-2-like properties (Chaouat *et al.*, 1990). (It is worth recalling that TGF-β 2 can block the proliferation/activation of the aforementioned lymphocyte subsets and, in addition, exert direct protective effects on targets of products from activated T and NK cells, exactly as does TNF, when tested on such targets as murine embryonic fibroblasts, or the murine malignant fibroblastic cell line, L929.) Although placenta-mediated suppression is deficient in resorbing animals on Day 11 (Chaouat *et al.*, 1985), no such difference is seen at earlier stages, whereas there is a clear correlation between the level of decidual suppression at the implantation site and subsequent fetal resorption (Clark *et al.*, 1987). It is therefore possible that a defect in local suppression would result in too high a level of local NK-cell activity, and recruitment and/or activation of LAKCs, thus leading to embryo demise.

Environmental factors are involved in the CBA × DBA/2 resorption models (Hamilton & Hamilton, 1987), most likely microbial in nature and probably resulting in local activation of non-specific effectors. IL-2 can lead to NK-cell differentiation into LAKCs, and it is likely that injections of excess IL-2 by-pass local active suppression and trigger such detrimental activation/differentiation. Our data are in keeping with those of Tezabwala *et al.* (1989), although in our hands we need to inject higher doses than they do to obtain induction of abortion. Such discrepancy is most probably due to environmental conditions, as discussed below for 'basal' resorption rates.

NK activation can be directly achieved in pregnant females by Poly(I).Poly(C12)U injection. Injection of spleen cells from animals Poly(I).Poly(C12)U into CBA/J females, or direct treatment of these, elicits abortion (Baines & de Fougerolles, 1988; Chaouat *et al.*, 1988c, d, 1989a, b). Such effects are seen not only in CBA/J, but also in BALB/c, C57BL/6 mice, which are non-resorption-prone strains (Chaouat *et al.*, 1989b, c, 1990; unpublished data). This paper demonstrates that the effects on pregnancy outcome of such activated NK cell-enriched cellular transfer can be mimicked by injecting TNF and γ-interferon, which are products of such activation. Their action could be cytostatic for placental cells, as demonstrated *in vitro*, or directly cytotoxic for non-trophoblastic components of the placenta (such an effect is highly likely in the case of embryonic fibroblasts), whereas IL-2 is likely to act by activating LAKCs themselves. In addition, TNF could act by causing local necrosis as a consequence of its action on the blood vessels that penetrate the placenta.

TNF could be produced as a result of local triggering of decidual T cells and NK cells, as well as macrophages, as a bystander effect of infection. LPS would merely mimic the infection-related event. Such a mechanism would account for environmental or ageing effects in the CBA/J × DBA/2 system (Hamilton & Hamilton, 1987; Chaouat *et al.*, 1986, 1988b). We took advantage of such effects to enhance the basal resorption rates in our CBA/J mice by letting them age for the R GM-CSF experiments. This effect accounts for most of the variation one can observe between our tables, since mice of the same age in the same room had no gross (statistically significant) variations in resorption rates throughout the year.

The effect of IL-3 is more complicated to explain. Such a therapeutic effect does not necessarily represent a physiologically significant event, because IL-3 has not yet been demonstrated at the maternal–fetal interface, although one report describes the presence of IL-3 mRNA in the decidua (Shorter *et al.*, 1989). Nevertheless, placental cells appear to be responsive *in vitro* to all members of the CSF family tested so far, and GM-CSF itself has been shown to have direct trophic effects on outgrowth of pure ectoplacental cone trophoblast (Armstrong & Chaouat, 1989).

GM-CSF can also be released from the decidua. The doses of recombinant murine GM-CSF that we inject are far too low to act directly on placental cell growth in the mouse, as deduced from in-vitro titration curves. Furthermore, human GM-CSF protects against high resorption rates in the murine CBA × DBA/2 matings. This lymphokine has no direct effect on murine placental cell growth *in vitro*, and human GM-CSF does not sustain the growth or promote colony formation by mouse progenitor cells. The involvement on placental or decidual cells of a new type of CSF receptor, able to mediate activities of this lymphokine on non-lymphoid progenitor, with no species restriction, has been suggested (G. Vadas, unpublished data) but not yet proved. The demonstration of GM-CSF receptor on human trophoblast, possibly a new receptor, could clarify this issue (Uzumaki *et al.*, 1989). In the meantime, to explain these effects, we suggest at least four possible mechanisms, which are not necessarily mutually exclusive, and deserve consideration for future experimentation. (1) GM-CSF could trigger local release of CSF1 or more GM-CSF by macrophages or other cells leading to a direct or indirect trophic effect. (2) CSF1 might be released by placental cells themselves in response to low doses of GM-CSF in a paracrine loop, and high doses would result in a negative feed back. (3) Since some T cell lymphomas have been shown to secrete CSF1 (Pralloran *et al.*, 1989), and since HILDA/LIF, a product of CD4 and CD8 T cells has a direct trophic effect on extra somatic cells (Moreau *et al.*, 1988), the possibility that GM-CSF might have an effect on such a T cell subset is worthy of further examination. (4) GM-CSF is involved with interferon in natural suppressor mechanisms (Cleveland *et al.*, 1988), which share a certain number of properties with decidua and placenta-associated suppression, and IL-3 can abrogate LAKC activation/generation in the human (Gallagher *et al.*, 1988). Using the measurement of TNF-α to assess cytotoxicity of decidual supernatant on the L929 malignant murine fibroblast cell line, a classical test of TNF activity, we observed an inverse correlation in the CBA × DBA/2 system between TNF and GM-CSF titres (assessed as HCSF activity) in the decidua (Chaouat *et al.*, 1990). Although L929 is sensitive to TNF-α and -β, as well as the cytotoxic effects of other lymphokines, it is tempting to speculate that placental growth is under the control of decidual stop signals, amongst which TNF production by activated natural killer cells and lymphokine-activated killer cells, as well as granulated metrial gland cells, could be an element. Down-regulation by members of the CSF family of lymphokines of TNF production would result in enhanced placental growth, which would be functionally indistinguishable from a direct trophic effect, and may operate in parallel with it.

Whatever the mechanism(s) involved the results described in this paper indicate a direct influence of lymphokines on reproductive outcome *in vivo*.

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References

Armstrong, D.T.A. & Chaouat, G. (1989) Effects of lymphokines and immune complexes on murine placental cell growth in vitro. *Biol. Reprod.* 40, 400–406.

Athanassakis, I., Bleackley, R.C., Paetkau, V., Guilbert, L., Barr, P.J. & Wegmann, T.G. (1987) The immunostimulatory effects of T cells and T cell lymphokines on murine fetally derived placental cells. *J. Immunol.* 138, 37–44.

Baines, M. & de Fougerolles, R. (1988) Modulation of Natural Killer activity influences resorption rates in CBA × DBA/2 matings. *J. Reprod. Immunol.* 11, 147–153.

Chaouat, G., Kiger, N. & Wegmann, T.G. (1983) Vaccination against spontaneous abortion in mice. *J. Reprod. Immunol.* 8, 389–394.

Chaouat, G., Kolb, J.P., Kiger, N., Stanislawski, M. & Wegmann, T.G. (1985) Immunological concomitants of vaccination against abortion in mice. *J. Immunol.* 134, 1594–1598.

Chaouat, G. (1986) Placental infiltration of resorbing CBA × DBA/2 embryos. *J. Reprod. Immunol., Suppl* 1, 134, Abstr.

Chaouat, G., Lankar, D., Kolb, J.P. & Clark, D.A. (1987) 2 modeles d'avortements d'origine immunitaire chez la souris de laboratoire: mecanismes abortifs, modalites et mecanismes du traitement par l'immunisation contre un male relie ou non relie suivant les differences antigeniques père mère. In *Immunologie de la Relation feto-maternelle* (Colloq. INSERM No. 154), pp. 243–255. Ed. G. Chaouat. INSERM, Paris.

Chaouat, G., Menu, E., Athanassakis, I. & Wegmann, T.G. (1988a) Maternal T Cells regulate placental size and fetal survival. *Regional Immunology* 1, 143–148.

Chaouat, G., Clark, D.A. & Wegmann, T.G. (1988b) Genetics aspects of the CBA/J × DBA/2 J and B10 × B10.A models of murine spontaneous abortions and prevention of leukocyte immunisation. In *Early Pregnancy Loss. Mechanisms and Treatment*, pp. 89–105. Eds W. R. Allen, D. A. Clark, T. J. Gill III, J. F. Mowbray & W. R. Robertson. RCOG Press, London.

Chaouat, G., Menu, E., Bustany, P., Rebut-Bonneton, C. & Wegmann, T.G. (1988c) Role du placenta dans le maintien de l'allogreffe fetale. *Reprod., Nutr. Develop.* 28, 1587–1588.

Chaouat, G., Menu, E., Wegmann, T.G., Clark, D.A., Minkowski, M., Dy, M. & Thang, M.N. (1988d) Explications actuelles du maintien de l'allogreffe fetale et hypothèses de travail sur le mécanisme d'effet des vaccins antiavortifs (Modèles murins). *Contraception Fertilité Sexualité* 17, 57–59.

Chaouat, G., Szekeres-Bartho, J., Menu, E., Kinsky, R., Thang, M.N., Dy, M. & Minkowski, M. (1989b) Placental interactions with maternal immune system. In *Experimental Models in Obstetrics and Gynaecology*. Ed. C. Romanini. Parthenon Publishing Ltd, London. In Press.

Chaouat, G., Menu, E., Kinsky, R., Dy, M., Minkowski, M., Delage, G., Thang, M.N., Clark, D.A., Wegmann, T.G. & Szekeres-Bartho, J. (1989c) Lymphokines and non specific cellular lytic effectors at the feto maternal interface affect placental size and survival. In *Reproductive Immunology, 1989*. Elsevier, Amsterdam. In Press.

Chaouat, G., Menu, E., Szekeres-Bartho, J., Rebut-Bonneton, C., Bustany, P., Kinsky, R., Dy, M., Minkowski, M., Clark, D.A. & Wegmann, T.G. (1990) Immunological and endocrinological factors that contribute to successful pregnancy. In *Molecular and Cellular Immunology of the Feto-maternal Interface* (in press). Eds T. G. Wegmann & T. J. Gill III. Oxford University Press.

Clark, D.A., McDermott, M. & Szewzuk, M.R. (1980) Impairment of host versus graft reaction in pregnant mice: (II) Selective suppression of Cytotoxic cell generation correlates with soluble suppressor activity and successful allogeneic pregnancy. *Cell. Immunol.* 52, 106–118.

Clark, D.A., Kiger, N., Guennet, J.L. & Chaouat, G. (1987) Local active suppression and successful vaccination against spontaneous abortion in CBA/J mice. *J. Reprod. Immunol.* 10, 79–86.

Clark, D.A., Falbo, M., Bruce-Rowley, R., Banwatt, D. & Stedronska-Clark, J. (1988) Active suppression of host versus graft reaction in pregnant mice. (IX) Soluble suppressor activity obtained from allopregnant mouse decidua that blocks the cytolytic effector response is related to TGF Beta. *J. Immunol.* 141, 3833–3841.

Clark, D.A., Head, J.R., Drake, B., Fulop, G., Brierley, J., Manuel, J., Barwatt, D. & Chaouat, G. (1990) Role of a factor related to transforming growth factor beta 2 in successful pregnancy. In *Molecular and Cellular Immunology of the Feto-maternal Interface* (in press). Eds T. G. Wegmann & T. J. Gill III. Oxford University Press.

Cleveland, M.G., Lane, R.G. & Klimpel, G.R. (1988) Spontaneous Interferon Beta production. A common feature of Natural Suppressor Systems. *J. Immunol.* 141, 2043–2048.

Croy, B.A. & Kussouf, S.A. (1989) Evaluation of the murine metrial gland for immunological function. *J. Reprod. Immunol.* 15, 51–71.

Croy, B.A., Wood, W. & King, G.J. (1987) Evaluation of intrauterine immune suppression during pregnancy in a species with epitheliochorial placentation. *J. Immunol.* 139, 1088–1095.

Drake, B.L. & Head, J.R. (1988) Murine trophoblast cells are susceptible to Lymphokine Activated killer (LAK) cell lysis. *Am. J. Reprod. Immunol.* 16, 114, Abstr.

Drake, B.L. & Head, J.R. (1989) Murine trophoblast cells can be killed by allospecific cytotoxic T lymphocytes generated in Gibco OPTI MEM medium. *J. Reprod. Immmol.* 15, 71–77.

Dy, M., Schneider, E., Gastinel, L.N., Aufray, C., Mermod, J.J. & Hamburger, J. (1987) Histamine Colony Stimulating Factor (HCSF) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) activities are borne by the same molecule. *Eur. J. Immunol.* 17, 1243–1247.

Gallagher, G., Wilcox, F. & Al-Azzawi, F. (1988) Interleukin 3 and Interleukin 4 each strongly suppress induction and function of human LAK cells. *Clin. exp. Immunol.* 74, 166–171.

Gendron, R.L. & Baines, M. (1988) Infiltrating decidual Natural Killer cells are associated with spontaneous abortion in mice. *Cell. Immunol.* 113, 261–265.

Gendron, R.L., Nestel, F.P., Lapp, W.S. & Baines, M.G. (1989) Lipopolysaccharide induced fetal resorption involves embryo associated production of Tumor Necrosis Factor. *Proc. 7th Int. Congr. Immunol., Berlin*, Abstr. 118-017.

Hamilton, M.S. & Hamilton, B.L. (1987) Environmental influences on immunologically associated recurrent spontaneous abortion in CBA/J mice. *J. Reprod. Immunol.* 11, 237–241.

Mowbray, J.F., Lidell, H., Underwood, J.L., Gibbs, S.C., Reginald, P.W. & Beard, R.W. (1985) Controlled trial of treatment of recurrent spontaneous abortions by immunisation with paternal cells. *Lancet* i, 941–943.

Parand, M. (1987) Role of TNF in non specific stimulation of mouse resistance to infection. *Immunobiology* 175 (1/2), 26, Abstr.

Parand, M. & Chedid, L. (1964) Protective effects of chlorpromazine against endotoxin induced abortion. *Proc. Soc. exp. Biol. Med.* 116, 906–915.

Moreau, J.F., Donaldson, D.D., Bennet, F., Witek Giannotti, J., Clark, S.C. & Wong, G.G. (1988) Leukemia inhibitory factor is identical to the myeloid growth factor for human interleukin for DA cells. *Nature, Lond.* 336, 6200, 690–692.

Pralloran, V., Gascan, H., Boursier, M.C. & Soulillou, J.P. (1989) Sécrétion de M-CSF/CSF/1 par un lymphome T. *Proc. French Soc. Immunol., Nantes* p. 49, Abstr.

Shorter, S.C., Vince, G.S. & Starkey, P.M. (1989) The identification of mRNA for the IL-3 related cytokines in placental and decidual tissues. *Proc. 3rd Mtg Eur. Placenta Group.* Eds L. Cedard, G. Chaouat, J. C. Challier & C. Neismann. INSERM, Paris. In press.

Taylor, C. & Faulk, W.P. (1981) Prevention of recurrent spontaneous abortions with leukocyte transfusion. *Lancet* ii, 68–70.

Tezabwala, B.U., Johnson, P.M. & Rees, R.C. (1989) Inhibition of pregnancy viability in mice following IL-2 administration. *Immunology* 67, 115–120.

Uzumaki, H., Okabe, T., Sasaki, N., Haghwara, K., Takaku, F., Tobita, M., Yasukawa, K., Ito, S. & Umezawa, Y. (1989) Identification and characterisation of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. *Proc. natn. Acad. Sci. USA.* 86, 9323–9326.

Wegmann, T.G., Athanassakis, I., Guilbert, L., Branch, D., Dy, M., Menu, E. & Chaouat, G. (1989) The role of M-CSF and GM-CSF in fostering placental growth, fetal growth, and fetal survival. *Transplantation Proc.* 21, 566–569.

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Effects of granulosa cell co-culture on in-vitro meiotic resumption of bovine oocytes

M. A. Sirard and S. Bilodeau

Département de Zootechnie, Université Laval, Québec, Canada G1K 7P4

Summary. This study was undertaken to create an in-vitro model using granulosa cell monolayers to replace the role of the follicle in the maturation of bovine oocytes. Cumulus–oocyte complexes were co-incubated with fresh or 7-day granulosa cell cultures (with new or conditioned medium) or with conditioned medium alone, in the presence or absence of IBMX (isobutylmethylxanthine), adenosine or heparin. Progression to the metaphase-II stage was significantly affected by the co-culture of oocytes with bovine granulosa cell monolayers and to a lesser degree when cultured with supernatant alone (conditioned medium). The oocytes attached rapidly to the monolayer, suggesting that the intimate contact between the granulosa cells and the cumulus–oocyte complexes is an important signal for the maintenance of meiotic arrest. Heparin did not prevent maturation itself, but prevented attachment of cumulus–oocyte complexes to monolayers, thereby reducing their inhibitory effect. Adenosine prevented cumulus expansion and reduced maturation and IBMX was an effective inhibitor only in the presence of additional granulosa cells.

Keywords: oocyte; meiosis; granulosa cells; *in vitro*; cattle

Introduction

Pincus & Enzmann (1935) showed that mammalian oocytes, once removed from the precise environment of the follicle, can resume the first meiotic division spontaneously in simple culture media. For cattle, information on the meiotic process is needed to enhance the developmental potential of in-vitro matured oocytes, since one possible cause of developmental defects is the incompetence of oocytes from the smaller follices. If germinal vesicle arrest could be maintained *in vitro*, it would be possible to influence both the cytoplasm and the nucleus with hormones or granulosa cells before nuclear maturation.

Bovine oocytes are not as sensitive as oocytes from other animals studied to protein kinase stimulation via cAMP accumulation (Sirard & First, 1988). Results obtained for cattle (Sirard & First, 1988) and sheep (Moor & Heslop, 1981) indicate that cAMP accumulation in the oocyte may not be the only physiological way to maintain the meiotic arrest in spite of the fact that bovine oocytes possess an active adenylate cyclase enzyme (Kuyt *et al.*, 1988) and respond temporarily to cAMP variations (Homa, 1988; Sirard & First, 1988). The effect of isobutylmethylxanthine (IBMX) in cows is similar to the effect of cAMP analogues and results in a delayed breakdown of the germinal vesicle (GVBD) followed by normal maturation or a metaphase-I block (Ball *et al.*, 1984; Sirard & First, 1988). If denuded oocytes are used, much greater amounts (5 mM, 25 times) of IBMX are required to inhibit GVBD (Homa, 1988). In the mouse, cAMP variations are modulated principally by phosphodiesterase inhibitors, and purines are therefore also effective inhibitors since their major effect is related to the inhibition of phosphodiesterase (Downs *et al.*, 1989). Purines have a weak effect on the meiotic resumption of bovine oocytes (Sirard & First, 1988) and since bovine follicular fluid does not contain amounts of purines comparable to those